Khalid Rehman Hakeem
Reiaz Ul Rehman
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Editors

Plant signaling: Understanding the molecular crosstalk

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Editors
Khalid Rehman Hakeem
Universiti Putra Malaysia
Serdang, Selangor, Malaysia

Inayatullah Tahir
Department of Botany
University of Kashmir
Srinagar, Jammu and Kashmir, India

Reiaz Ul Rehman
Department of Bioresources
University of Kashmir
Srinagar, Jammu and Kashmir, India

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Foreword

Through the ages, plants have evolved and used intricate networks of interacting molecules for regulating growth and propagation and also to mount defence mechanisms to overcome and adapt to adverse conditions brought about by abiotic (environmental) and biotic (pathogenic) stresses. In recent times, environmental stresses have intensified due to global warming and climate change-induced adverse conditions such as salinity, submergence, drought and temperature fluctuations. Due to loss of biodiversity and over-reliance on chemicals, there has also been an increase in infection-related stresses. These have had an adverse effect on agricultural production. To ensure food security, it may be necessary to intervene genetically to speed up the defence and adaptation processes. For this, it is very important to understand the molecular mechanisms that underlie defence and adaptation to biotic and abiotic stresses. This new book "Plant Signaling: Understanding the Molecular Crosstalk" will be a very good introduction to the subject.

The environmental and pathogenic stress signals are recognized by stress-specific sensor proteins embedded in the plant cell membrane that help to transmit the exogenous signals from the cell surface to the nucleus where they stimulate different regulatory genes and transcription factors responsible for the expression of specific protective and adaptive proteins. Plant infection triggers production of resistance proteins that bind to pathogen-derived factors. This interaction initiates a signal transduction cascade that ultimately causes localized cell and tissue death at the site of infection and imparts non-specific immunity to the rest of the cell. Understanding the host-pathogen interactions at the molecular level could help with the engineering of disease-resistant transgenic crops.

"Plant Signaling: Understanding the Molecular Crosstalk" is a very timely and relevant anthology of review chapters that outline the current understanding of the myriad ways in which different molecules interact directly and indirectly within various regulatory networks to provide protection and adaptive capability to plants. Different chapters of the book review the current knowledge of different exogenous and intracellular factors involved in stress recognition, signal transduction and expression of genes involved in disease resistance and immunity and the activation of specific transport channels and adaptive molecules. The references will be particularly helpful in guiding researchers and students to original literature that provide information on the various molecular crosstalks that help the plants to adapt to adverse conditions and infections.

Some of the signalling cascades and signalling molecules described in detail include cyclic nucleotide-gated channels involved in multiple pathways related to immunity and stress, plant-specific *Rab* GTPases involved in membrane trafficking and several pathways and reactive oxygen species (ROS) which in optimum amounts help to overcome external stresses but in larger amounts can be harmful to plants. A number of chapters provide details of cell signalling pathways that could help plants to adapt to salinity, drought, heat and cold stress. Plant sugars could also be involved in cell signalling through interaction with plant stress hormones such as ethylene. A number of chapters also demonstrate how plants utilize a large number of signalling molecules and pathways for their own survival, growth and maintenance of biodiversity. These include membrane-active phosphoinositols and "SNARES", the pathways involved in self-incompatibility (for rejection of self-pollen), senescence, nitrogen regulation and signalling and symbiotic and nonsymbiotic associations between plants, fungi and bacteria.

Unravelling in detail the *N*-signalling pathway could result in the construction and production of high *N*-efficient genotypes of important food crops. Understanding the molecular signalling mechanisms and the functional genomics of important agricultural crops and also of wild-type plants and organisms with important resistance traits could help in the identification of novel pathogenic and stress-resistance genes that could be useful in the production of useful transgenic plants resistant to infections or adapted to climate change-induced stresses. "Plant Signaling: Understanding the Molecular Crosstalk" will be a very valuable addition to academic libraries and a handy source of information to researchers involved in research and innovation in the field of food security and biodiversity.

Incepta Visiting Professor Centre
for Advanced Research in Sciences,
Dhaka University, Dhaka, Bangladesh

Ahmed Abdullah Azad

Formerly:
Chief Research Scientist, CSIRO, Melbourne, Australia
Director of Research, University of Cape Town, South Africa
Member, Council of Scientific Advisors, International
Centre for Genetic Engineering and Biotechnology
Member, Board of Directors, Medical Research Council, South Africa

Preface

The ever-growing population of the world will have an adverse effect on the agricultural productivity. Besides, many other factors are responsible for a decline in crop productivity including the over-exploitation of bioresources, mal-agricultural practices and deleterious abiotic environmental stresses. For meeting the increasing food demands and overcoming the losses incurred by various factors, researchers are always on the look for the development of novel strategies. The new scientific innovations are reached upon through interdisciplinary approaches and by broadening the research area. Plants have unique abilities to grow in varied locations all over the world, and they show remarkable developmental plasticity for sustaining in these continually changing environments. Unlike other organisms, plants are sessile and they experience a plethora of biotic as well as abiotic stresses such as pathogens (viral, bacterial, insects, etc.), drought, salinity, temperature and metal toxicity. The plants have adapted themselves against these stresses by developing specialized mechanisms. They respond to the external cues by changing their morphologies, and these signals are recognized with the help of some membrane protein sensors that in turn are transduced to the nucleus. Ultimately, the nuclear transcription factors and genes are stimulated to form the product leading to plant adaptations at various levels and thus assisting them to sustain and surpass the adverse conditions.

Over the last 20 years, the concept of signalling is being continually modified, especially the molecular crosstalk associated with it. It has taken a shape of a new field due to the complexities and broadening of the knowledge base in the subject. Furthermore, during the last 10 years, the understanding in the field of plant signalling has been greatly enhanced by the development of various bioinformatic tools. The mapping of various plants especially the *Arabidopsis thaliana* has greatly assisted in identification of hormonal, developmental and environmental signal transduction pathways and the existing crosstalk between them. Signalling pathways utilize a complex network of interactions to orchestrate biochemical and physiological responses such as flowering, fruit ripening, germination and photosynthetic regulation, besides shoot or root development. Signalling agents that appear to be common to many different pathways include Ca^{2+}, inositol phospholipids-proteins, cyclic nucleotides, protein kinases and protein phosphatases. Any signal transduction pathway may involve the coordination of multiple signalling agents operating in multiple cellular structures. These agents never work in isolation, but always in networks that intersect multiple signalling pathways. The hormone-mediated

pathways (GA, IAA, ABA, JA, Br and SA) are involved in developmental responses. The cyclic nucleotide-gated channels (CNGCs) in plants are a part of complex signalling network that conduct cations and are responsible for diverse responses from stress tolerance, transpiration and fertilization. Phosphoinositides (PIs) comprise a family of minor membrane lipids which play important roles in many signal transducing pathways in the cell. Signalling through various PIs has been shown to mediate cell growth and proliferation, cytoskeleton organization, vesicle trafficking, regulation of ion channels and nuclear signalling pathways in various eukaryotic models. Membrane trafficking is a highly regulated process in which various molecular machineries are involved. It involves vesicle formation, tethering and finally fusion. SNAREs are involved in these membrane-trafficking events, and these interact with several proteins such as *Rabs* that act as regulators of SNARE-complex formation, and together they gate channels' potential signalling processes. Amongst the environmental factors which are involved in signalling are the reactive oxygen species (ROS) generated during cell metabolism. ROS are spontaneously produced in the cell by auto-oxidation reactions. Some of these ROS are physiologically useful and in fact necessary for life but can also be harmful if present in excess or in inappropriate amounts. The NO-mediated pathways have a role in plant defence and immunity. The cold stress tolerance mechanisms involve the sensing followed by expression of cold-responsive genes and transcription factors leading to formation of compatible solutes or osmoprotectants. The environmental factors are also responsible for the initiation of senescence process that is coordinated through common signalling networks involving various phytohormones acting as signalling molecules (ethylene, ABA, SA and JA). The biotic stresses (pathogens) constantly challenge the plant defence, and the disease development is rare due to resistance mechanisms which are interconnected signalling networks.

The plant signalling is an ocean of information, and it is a challenge to comprehend and cover the entire aspects of a subject at the same place. The present book is providing the detailed coverage of a broad range of aspects of signalling and emphasizing the critical importance of such signalling to plant and associated systems.

Serdang, Malaysia — Khalid Rehman Hakeem
Srinagar, India — Reiaz Ul Rehman
Srinagar, India — Inayatullah Tahir

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Plant Signalling: Response to Reactive Oxygen Species

1

Bisma Malik, Tanveer Bilal Pirzadah, Inayatullah Tahir, Reiaz Ul Rehman, Khalid Rehman Hakeem, and M.Z. Abdin

Abstract

It is noteworthy to mention how the last 20 years have modified the concept of signalling in plants, especially the molecular crosstalk associated with it. Plants have the ability to show remarkable developmental plasticity to sustain in a continually changing environment. In response to various environmental stresses such as drought, salinity, metal toxicity, temperature and pathogens, plants defend themselves by developing some special defence mechanisms. Plants recognise these environmental signals with the help of some membrane protein sensors and then transduce these signals to the nucleus which ultimately stimulates various transcription factors and genes to form the product that ultimately leads to plant adaptation and assists the plant to sustain and surpass the adverse conditions. Amongst the environmental factors which are involved in signalling is the reactive oxygen species (ROS) generated during cell metabolism. ROS are spontaneously produced in the cell enzymatically through the action of various soluble membrane-bound enzymes and nonenzymatically by autoxidation reactions. Some of these ROS (e.g. superoxide dismutase, hydrogen peroxide and nitric oxide) are physiologically useful and in fact necessary for life but can also be harmful if present in excess or in inappropriate amounts. Current research in this regard focuses more on the

B. Malik • T.B. Pirzadah • R. Ul Rehman (✉)
Department of Bioresources, University of Kashmir, Srinagar 190006, Jammu and Kashmir, India
e-mail: bisma767@gmail.com; pztanveer@gmail.com; pztanveer@yahoo.com; reiazrehman@yahoo.co.in

I. Tahir
Plant Physiology Lab, Department of Botany, University of Kashmir, Srinagar 190006, Jammu and Kashmir, India
e-mail: inayatullahtahir@gmail.com

K.R. Hakeem
Faculty of Forestry, Universiti Putra Malaysia, Serdang 43400, Selangor, Malaysia
e-mail: kur.hakeem@gmail.com

M.Z. Abdin (✉)
Center for Biotechnology, Jamia Hamdard, New Delhi 110062, India
e-mail: mzabdin@rediffmail.com; mzabdin@jamiahamdard.ac.in

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development of transgenic plants with enhanced tolerance to ROS by using genetic approaches and analytical techniques. In particular nitric oxide (NO), a reactive radical, may be involved in the defence mediated by the ROS such as defence gene activation, hypersensitive response cell death and phytoalexin biosynthesis. By using biotechnological approaches NO together with ROS activates a stronger response and tolerance to various stresses in plants.

Keywords
Signal transduction pathways • Defence mechanism • ROS • Oxidative stress • NO • Transgenic plants

1.1 Introduction

Plants are sessile organisms and are constantly being exposed to a plethora of biotic as well as abiotic stress conditions such as temperature, drought, heavy metal and salinity stresses (Mahajan and Tuteja 2005; Hakeem et al. 2012) (Figs. 1.1 and 1.2). These stress conditions greatly influence the plant growth, development, distribution and productivity. Besides, these stress conditions influence various metabolic reactions that lead to the production of different toxic compounds such as ROS formation which have deleterious effects on the physiology of plants. However during the course of evolution, plants have developed an array of mechanisms manifested through modified physiology to sustain environmental abiotic stress and resist hurdles originating from biotic stress. Plant acclimatisation to abiotic stress conditions usually depends upon the stimulation of cascades of molecular channels involved in stress perception, signal transduction and the expression of particular stress-related genes and metabolites (Guo-Tao et al. 2012). These abiotic as well as biotic signalling agents typically bring about their effect by means of sequences of biochemical reactions, termed as signal transduction pathways, that greatly amplify the original signal and ultimately result in the stimulation or repression of genes. Signal transduction is the term generally used to define the distinct array of biochemical mechanisms that control cellular physiology. The term "signal transduction" became attractive in the early 1980s, and now it is considered to be one of the most intensively studied areas that is spawning increasing interest worldwide. Although much information is known about the animal and fungal signal transduction pathways, analysis of signalling pathways in plants has come from since the pioneering studies which identified phytochrome as the first receptor in plants. These phytochromes were the only known receptors in plants until the early 1990s when a number of putative receptors were identified. Signal fluctuations vary from time to time both qualitatively and quantitatively. However, these signals are carried out by various cellular components such as xylem and phloem, the circulatory system, which can accommodate very large and rapid fluxes. The two fundamental components of signal transduction pathways involve intracellular Ca^{+2} and protein kinases, enzymes that phosphorylate and thereby alter the function of target proteins. Changes in protein phosphorylation patterns were observed after exposure of plant cells to abiotic as well as biotic factors like temperature stress, fungal elicitors and hormones (Felix et al. 1991; Raz and Fluhr 1993) and during establishment of freezing tolerance (Monroy et al. 1993). Recently, progress has been made in identifying primary signal reception mechanisms and early events in signalling cascades in higher plants. Intracellular signalling proteins and second messengers are often used to characterise a readily diffusible molecule involved in transmitting signals from an extracellular source to the

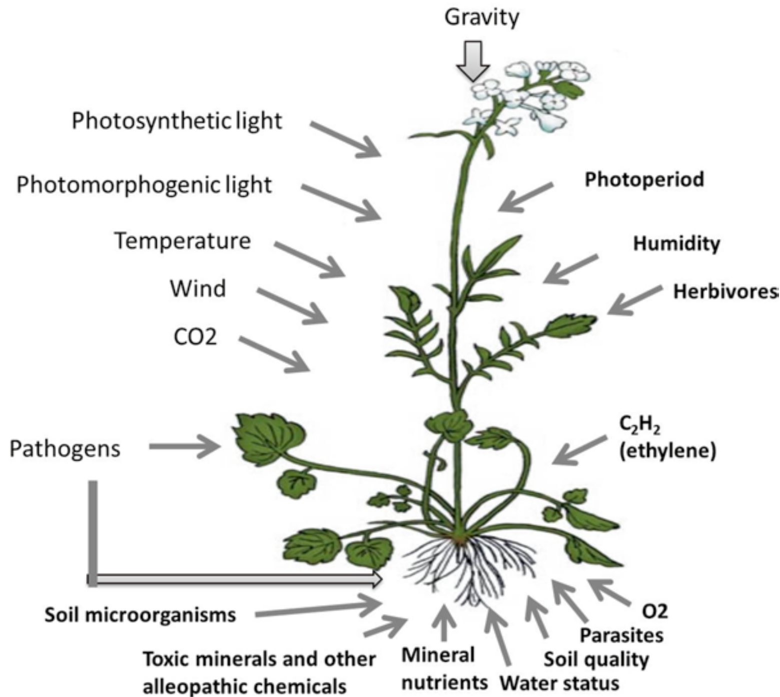


Fig. 1.1 External signals that affect plant growth and development include many aspects of the plant's physical, chemical and biological environments. Some external signals come from other plants. Apart from gravitropic signals, all other signals vary in intensity, often from minute to minute

premier target enzymes within the cell. In plants, cytosolic Ca^{+2} transduces many signals and is an eminent second messenger. Thus, it must be retained in the cytoplasm at concentrations many orders of magnitude lower than the Ca^{+2} in the cell wall. Ion channel-mediated signal transduction in higher plants has notable differences from signalling mechanisms in animal systems. Of the many types of ion channels found in higher plants, recent findings have indicated that an ion channels, along with Ca^{2+} channels, play critical and rate-limiting roles in the mediation of early events of signal transduction. Signals are mostly perceived membrane proteins, and therefore transmembrane events are the likely route for signal generation and transduction. In plants, the well-characterised plasma membrane-based receptors include transmembrane receptor enzymes (usually kinase), ROS sensors and G-protein-coupled receptors (GPCRs). Currently in plants, the G-protein-coupled receptors (GPCRs) are reported to be involved in processes such as ion channel and abscisic acid signalling and modulation of cell proliferation (Wang et al. 2001). Moreover, the signal transduction pathways in plants under abiotic stresses have been categorised into three main categories: (1) osmotic/oxidative stress signalling that involves mitogen-activated protein kinase (MAPK) modules, (2) Ca^{+2}-dependent signalling that leads to activation of LEA-type genes such as dehydration responsive elements (DRE)/cold-responsive sensitive transcription factors (CRT) class of genes and (3) Ca^{+2}-dependent salt overly sensitive (SOS) signalling that results in ion homeostasis (Xiong et al. 2002). In particular, current progress in this area has emphasised the role of Ca^{+2}- and Ca^{+2}/CaM-regulated transcription in plant cell response to stresses (Reddy et al. 2011). The two main objectives of engineering signalling pathways are to understand how natural networks function and to build synthetic

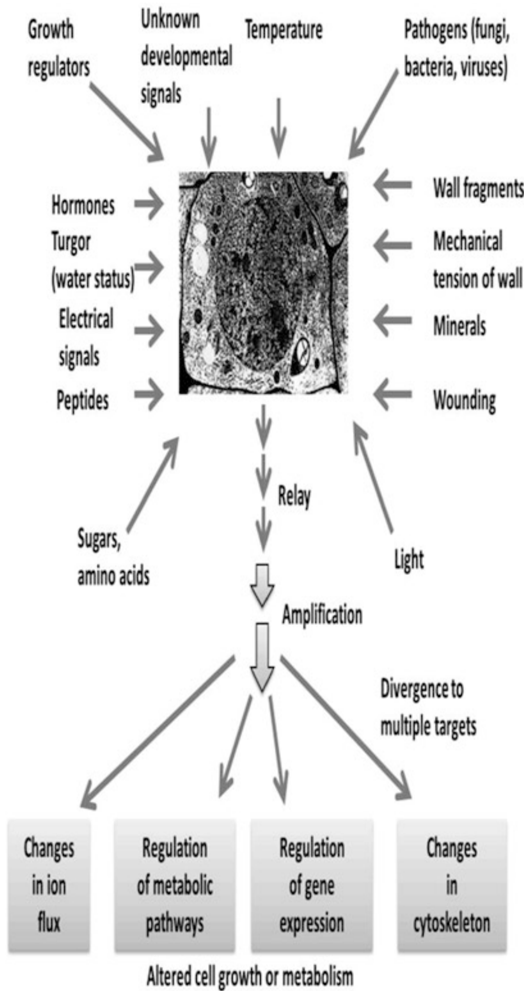


Fig. 1.2 A variety of internal signals modify plant cell metabolism, growth, and development. The ability of cells to respond to these signals is not confined to cells that are still growing and developing. Mature cells, too, can initiate metabolic responses and can even reinitiate growth and division in response to signal information

networks with specific applications or functionalities. Understanding the knowledge about the specificity of the stimulus, the biochemical nature of the receptor and the specificity of the responses is of paramount importance to better understand the diverse signalling pathways in plants. The main aim of this review is to discuss the mechanisms of signalling in plants, the ROS and NO signalling processes and their components, which commence thereafter and the resulting tolerance mechanisms.

1.2 General Features of Signal Transduction

The Signal transduction pathways are remarkably specific and exquisitely sensitive. The interaction between signal and receptor molecules is specific which happens due to complementarity amongst them. Specificity is achieved by precise molecular complementarity between the signal and receptor molecules (Fig. 1.3a). This process is mediated by the same kinds of weak (non-covalent) forces that mediate enzyme-substrate and antigen-antibody interactions. In the multicellular organisms the specificity is provided only by certain types of cells which contain the signalling receptors and intracellular targets of the signalling. Various genes are involved with the signalling process that are necessary for the plant cell to maintain the fate of development in the leaves and roots. A chain of various signalling molecules must be present within the transduction mechanism for the development of the cell because cells undergo changes during growth and they must maintain the internal and external conditions properly even in chaotic environmental conditions. The plant signalling pathway involves the "transducons" that are likely to be Ca^{2+} receptors such as calmodulin, calmodulin-binding proteins (e.g. kinase or microfilament-organising proteins) and calcium-dependent protein kinases. In single cell, the response of the entire plant must not be neglected. The plant cells and tissues individually require the co-ordination between them and complex mechanism of signal communication. Proteins, peptides and RNAs, growth factors, modulation factors, sugar and the mechanical signals are involved in tissues and cell communication (Trewavas 2002). The factors which are responsible for the sensitivity of signal transducers include receptors affinity for signal molecules, cooperativity in the ligand-receptors interaction and finally signal amplification by enzyme cascades.

Cooperativity in receptor-ligand interactions results in large changes in receptor activation with small changes in ligand concentration. Amplification by enzyme cascade results when

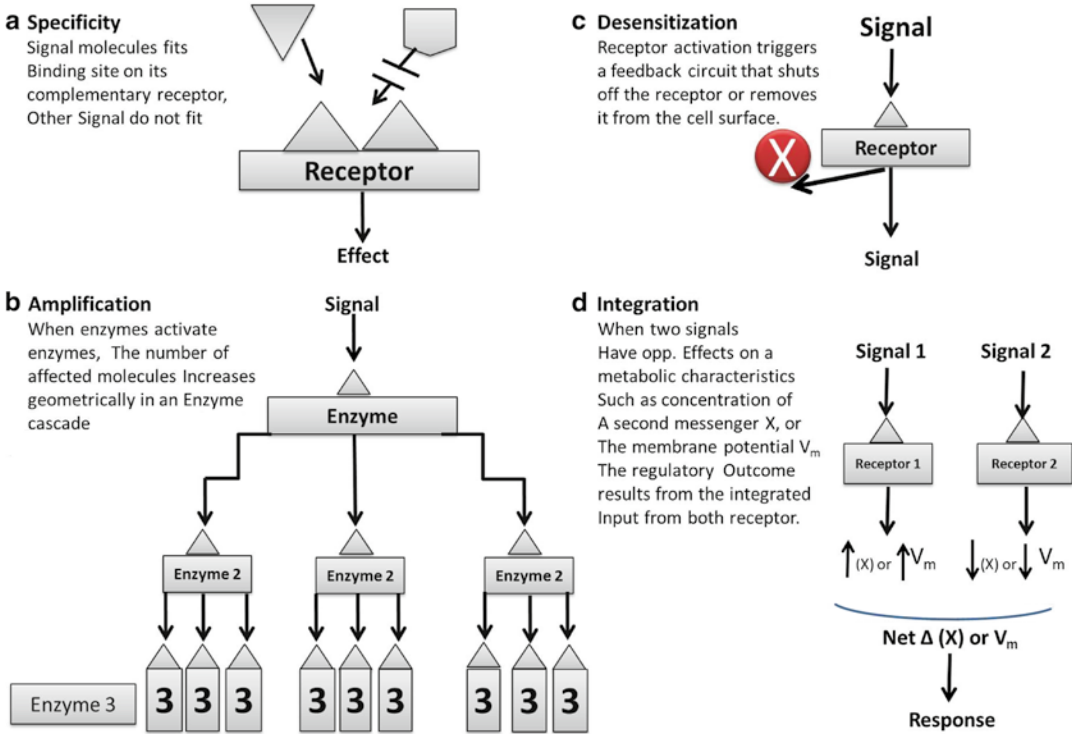


Fig. 1.3 Four features of signal-transducing systems (**a**) *Specificity*: Signal molecules fits binding site on its complementary receptor, Other signal do not fit. (**b**) *Amplification*: When enzymes activate enzymes, The number of affected molecules increases geometrically in an enzyme cascade. (**c**) *Desensitization*: Receptor activation triggers a feedback circuit that shuts off the receptor or removes it from the cell surface. (**d**) *Integration*: When two signals have opp. Effects on a metabolic characteristics such as concentration of a second messenger X, or the membrane potential $V_{m.}$ The regulatory outcome results from the integrated input from both receptor

an enzyme associated with a signal receptor is activated and, in turn, catalyses the activation of many molecules of a second enzyme, each of which activates many molecules of third enzyme and so on (Fig. 1.3b). Such cascade can produce amplifications of several orders of magnitude within milliseconds. The response to a signal must also be terminated such that the downstream effects are in proportion to the strength of the original stimulus. The sensitivity of a receptor system is subject to modification. When a signal is present, continuous desensitisation of the receptor system results (Fig. 1.3c); when the stimulus falls below a certain threshold, the system again becomes sensitive. A final noteworthy feature of signal-transducing systems is integration (Fig. 1.3d), the ability of the system to receive multiple signals, and produces a unified response appropriate to the needs of the cell or organism. Different signalling pathways converse with each other at several levels, generating a wealth of interactions that maintain homeostasis in the cell and the organism.

1.3 Role of Signal Transduction in Plants

Signal transduction in plants plays an essential role to combat multiplex of environmental and hormonal stresses. The abiotic and biotic stresses such as salinity, drought, temperature, pathogens and water stress possess ill effects on plant growth and development. In order to defend themselves, plants have developed some endogenous defence mechanisms to counteract such

stresses. Plants have the unique ability to cope up these stresses and survive under various stressful conditions. In this mechanism, the signals are initially perceived by various membrane-bound sensors which in turn get amplified and then transduce these signals to the nucleus in order to stimulate the cascade of reactions that ultimately lead the cell to function in the appropriate manner. Although there are various characteristics that make the plant resistant to environmental changes, some plants show plasticity towards these factors that adds an extraordinary layer of molecular and biochemical complexity that is a unique character of plants. There are many other growth substances like cytokinin, abscisic acid, ethylene and gibberellic acid, brassinosteroids, jasmonates and peptide hormone that act as agents in the signalling pathway and as such help to enhance the plasticity in plants. There are at least 600 receptor kinases present in *Arabidopsis,* and the majority of the receptor kinases are membrane bound. The receptor kinase in the plants plays an important role in incompatibility and disease defence signal transduction. The mechanism of signal transduction in plants involves many kinase cascades and transcription factors that regulate and complete the signalling pathway. A better knowledge of signalling/response coupling mechanisms in plants includes recognition of the stimulus by protein sensors that activates the gated ion channels like G-protein-coupled receptors (Fairchild and Quail 1998; Jones et al. 1998; White 2000), and generation of second messenger by hydrolysis of membrane components, viz. Phosphoinositides (Sanders et al. 1999; Poovaiah et al. 1999). Besides, it also involves enzyme stimulation especially protein kinases and transient increase in calcium influx. Moreover, phosphorylation and dephosphorylation of proteins is a ubiquitous mechanism to maintain the biological function of the cell. Recent progress has been achieved in identifying the signalling mechanism in higher plants. It has been revealed that the gated ion channels along with second messengers play an essential role in mediating intracellular responses in higher plant signal transduction. Through various studies it has been found that anion channels along with Ca^{2+} sensors, MAP kinases and ROS play crucial and rate-limiting roles in mediating plant responses and is now an emerging field of scientific research that finds important application in biotechnology and agriculture (Yinong et al. 2012). Some of the components that play a crucial role in plant signal transduction with recent advancements are as follows:

1.4 Role of ROS in Plant Signal Transduction

Free radicals which are defined as independent chemical species with one or more unpaired electrons can be formed from a diverse group of chemicals and are generally considered to be highly reactive. The term reactive oxygen species (ROS) is now generally used to denote all the oxygen-centred radicals as well as compounds containing chemically reactive oxygen functional groups such as singlet oxygen, hydrogen peroxide, hypochlorous acid and peroxide, hydroperoxide and epoxide metabolites of endogenous lipids and xenobiotics (Giri et al. 1999). ROS are generated spontaneously in a living cell during several metabolic pathways including biological electron transport system (photosynthetic, mitochondrial, microsomal), various enzymes and biomolecules: xanthine oxidase, cyclooxygenase, lipoxygenase, autoxidation of catecholamines etc. (Chandna et al. 2012; Halliwell 1999). Regulation of the multiplex redox and ROS signals in plants requires a high degree of co-ordination and balance between signalling and metabolic pathways in different cellular components (Nobushiro et al. 2012). The process of oxidation occurs in any oxygen-rich environment where substrates are exposed to light and heat. These processes are all mediated by highly reactive oxygen radicals (Harman 2000). During physiological processes, oxygen is involved in oxidation of substrates to generate energy which results in the production of oxygen radical. They also have a beneficial role in phagocytes where they protect the cell against bacteria and parasites. Various ROS such as superoxide radical, hydrogen peroxide and hydroxyl free radical can cause

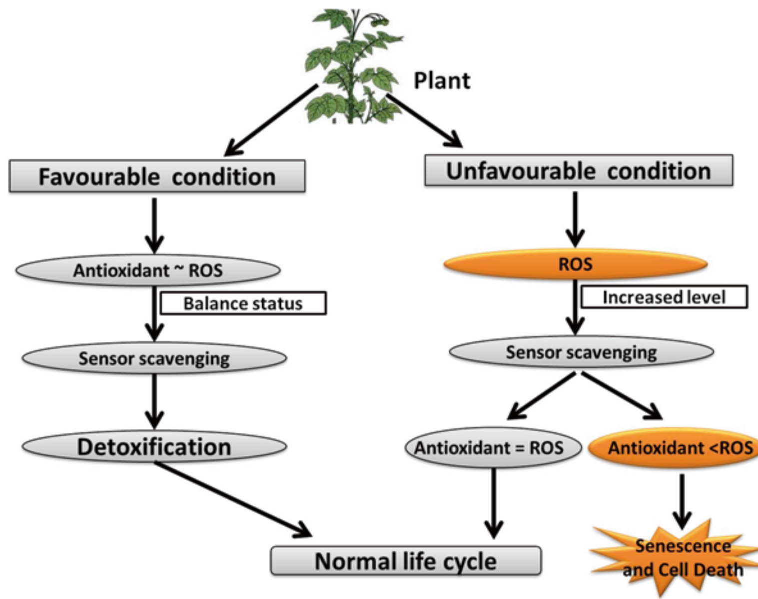


Fig. 1.4 Antioxidants and redox signaling in plants

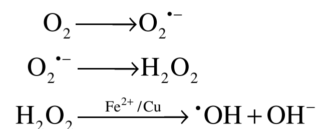
damage to respiring cells. These ROS are highly toxic, mutagenic and reactive (Halliwell 1997). The production of ROS is a physiological process due to oxidative metabolism of the cell. A serious imbalance between reactive oxygen species and antioxidants causes oxidative stress. Oxidative stress is caused by antioxidant deficiency or by increased production of reactive oxygen species (Fig. 1.4); by environmental stresses such as toxins, light and pathogens; or by inappropriate activation of defence responses (Halliwell 1997). Extreme production of ROS or inappropriate removal leads to oxidative stress which results in the malfunctioning of various physiological processes and damage to biological macromolecules (Chopra and Wallace 1998). In order to combat oxidative stress, plants exhibit an internal defence mechanism such as antioxidants: enzymatic or nonenzymatic or low molecular weight antioxidants. If ROS formation is high and antioxidant level is low, it results in the accumulation of free radical molecules in the cell, causing oxidative stress (O'Brien et al. 2012). The generation of free radicals and oxidative stress has been found to be involved as factors in development of a large number of diseases (Gambhir et al. 1997). Majority of diseases caused by the oxidative stress involves generation of not only ROS but also nitrogen reacting species (NRS) including free radicals (Beckman and Ames 1998; Gutteridge and Halliwell 2000). Besides having an important role in intra- and extracellular signalling, these reactive molecular species may start damaging metabolic reactions (Halliwell 1999). It has been found that oxidative stress may be responsible for biochemical events that results in the cancer formation such as activation of oxidative DNA damage and alternations in intracellular signal transduction (Allen and Tresini 2000; Martin and Barett 2002). Environmental sources such as ultraviolet irradiation, ionising radiations and pollutants also produce ROS (Halliwell 1997), and injured cells and tissues can activate the production of free radicals (Spiteller 2001) in plants. ROS can be formed in foods via lipid oxidation and photosensitisers exposed to light (Boff and Min 2002). In biological systems, ROS can be formed by pro-oxidative enzyme systems, lipid oxidation, irradiation, inflammation, smoking, air pollutants and glycoxidation (Steif 2003). As the plants are exposed to different environmental conditions such as biotic and abiotic

stresses including pathogens attack which results in the enhanced production of ROS in plants due to breakdown of cellular homeostasis (Srivastava and Dubey 2011). When the cell is said to be in a state of oxidative stress and the ROS level is enhanced considerably in response to environmental stresses, it results in the lipid peroxidation, oxidation of proteins, DNA damage, enzyme inhibition and activation of programmed cell death (PCD) pathway that ultimately leads to apoptosis (Mishra and Dubey 2011). Irrespective of their destructive activity, ROS are considered to be the second messengers in the cellular process including tolerance to environmental stress (Yan et al. 2007). Depending upon the equilibrium between ROS and antioxidant scavenging activity that whether ROS act as damaging or signalling molecules, the equilibrium between ROS and scavenging activity is very necessary to maintain by the cells in order to combat any oxidative stress. The scavenging activity is achieved by the antioxidant defence system that comprises enzymatic and nonenzymatic antioxidants (Pallavi et al. 2012). The enzymatic antioxidant defence system includes superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPX), glutathione reductase (GR), whereas ascorbate (ASA), glutathione (GSH), carotenoids, tocopherols and phenolics serve as potential nonenzymatic antioxidants within the cell (Noctor and Foyer 1998). Recent studies have reported that the oxidative stresses in plants system are decreased by increasing the activity of enzyme of the antioxidant defence system which is only possible by maintaining an extreme antioxidant activity to quench the toxic ROS and which in turn increases the plants tolerance to environmental stress (Q. Chen et al. 2010). In order to improve the stress tolerance of crops against environmental stress, considerable efforts have been made by developing transgenic lines with altered levels of antioxidants (Faize et al. 2011). Multiple expression of antioxidant enzyme than single or double expression has been found more effective for developing transgenic plants (Table 1.1) with enhanced tolerance to environmental stresses which in turn results in the increased crop productivity in agriculture (Lee et al. 2007).

1.4.1 Types of ROS, Their Generation and Effects

ROS are generated at various cellular compartments in the cell like chloroplast, mitochondria, plasma membrane, peroxisomes, apoplast, endoplasmic reticulum and cell walls under both stressed and unstressed cells (Fig. 1.5). Reactive oxygen species can be classified into oxygen-centred radicals and oxygen-centred non-radicals. Oxygen-centred radicals are superoxide anion ($O_2^{\bullet -}$), hydroxyl radical (•OH), alkoxyl radical (RO·) and peroxyl radical (ROO•), whereas oxygen-centred non-radicals are hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$). Other ROS are nitrogen-containing species such as NO• and nitric dioxide (NO_2) and peroxynitrite ($OONO^-$) (Simon et al. 2000; Huang et al. 2005).

These ROS are generated due to the leakage of electrons onto O_2 from the electron transport chain or as an output of several biochemical pathways localised in various cellular compartments. These are five possible species: superoxide anion radical ($O_2^{\bullet -}$), hydroperoxyl radical $\left(HO_2^{\bullet}\right)$, peroxide ion $\left(HO_2^{-}\right)$, hydrogen peroxide (H_2O_2) and hydroxyl radical (·OH).


$$O_2 \longrightarrow O_2^{\bullet -}$$
$$O_2^{\bullet -} \longrightarrow H_2O_2$$
$$H_2O_2 \xrightarrow{Fe^{2+}/Cu} {}^{\bullet}OH + OH^-$$

The $O_2^{\bullet -}$ and H_2O_2 so formed in presence of metal catalyst such as Cu^+/Fe^{2+} may lead to formation of most reactive •OH. Synthesis of $O_2^{\bullet -}$ and H_2O_2 leads to the generation of •OH which oxidises lipids and results in lipid peroxidation (Chessman and Slater 1993). A study described the role of H_2O_2, $^1O_2$, $O_2^{\bullet -}$ and the products of lipid peroxidation signalling molecules in the processes of stress signalling transduction in plants and also suggests that the redox regulators such as protein kinases/protein phosphatases and transcription factors play a critical role in the functioning of ROS-dependent signalling system in the plants (Kreslavski et al. 2012). Some of the reactive oxygen species are briefly discussed below:

Table 1.1 Genetic engineering approaches to achieve cold tolerance by over expression of antioxidant enzymes

Enzyme	Reaction catalysed	Transgenic plant against cold stress	References
Super oxide dismutase (SOD)	$O_2^- + O_2^- + 2H^+ \leftrightarrow 2H_2O + O_2$	Cu/Zn SOD from *Pisum sativum* to *Nicotiana*	Gupta et al. (1993)
		Fe-SOD from *A. thaliana* to *Medicago sativa*	McKersie et al. (2000)
		Mn-SOD in *Gossypium hirsutum*	Payton et al. (2001)
Catalase (CAT)	$2H_2O_2 \leftrightarrow 2H_2O + O_2$	Rice (*Oryza sativa*)	Matsumura et al. (2002)
Glutathione reductase (GR)	$NADPH + GSSG \leftrightarrow NADP + 2GSH$	Tobacco (*N. tabacum*)	Le Martret et al. (2011)
		From *A. thaliana* to *Gossypium hirsutum*	Payton et al. (2001) and Kornyeyev et al. (2003b)
Dehydro-ascorbate reductase (DHAE)	$AA + H_2O_2 \leftrightarrow DHA + 2H_2O$	From human to Tobacco (*N. tabacum*)	Kwon et al. (2003)
		Tobacco (*N. tabacum*)	Le Martret et al. (2011)
Ascorbate peroxidase (APX)		From *Pisum sativum* to *Gossypium hirsutum*	Kornyeyev et al. (2001, 2003a, b)
		From *Spinacia oleracea* to *N. tobaccum*	Yabuta et al. (2002)
		From *Pisum sativum* to *Lycopersicum esculentum*	Wang et al. (2005)
		Tomato (*Lycopersicum esculentum*) *StAPX* gene in Tobacco (*N. tabacum*)	Sun et al. (2010)

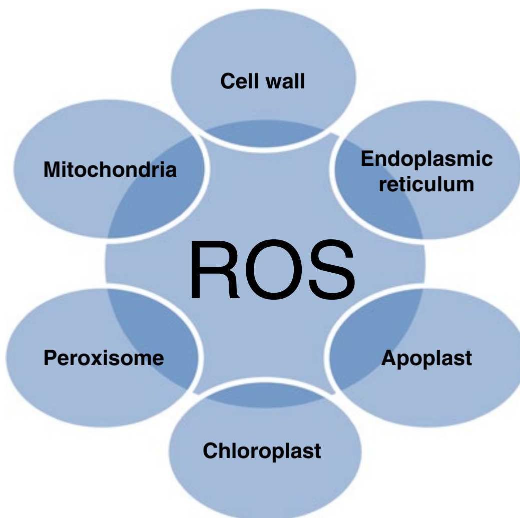
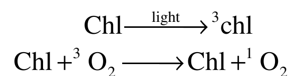


Fig. 1.5 Sites of production of reactive oxygen species (*ROS*) in plants

1.4.1.1 $^1O_2$

In the reaction centre of photosystem II, the highly reactive $^1O_2$ can be generated via triplet chlorophyll (Chl) production in the antenna system in the presence of light (Krieger-Liszkay 2005). The Chl triplet state can react with $^3O_2$ to give up very highly reactive ROS$^1O_2$



$$\mathrm{Chl} \xrightarrow{\text{light}} {}^3\mathrm{chl}$$
$$\mathrm{Chl} + {}^3\,O_2 \longrightarrow \mathrm{Chl} + {}^1\,O_2$$

$^1O_2$ formed thus reacts with biomolecules and directly leads to the oxidation of proteins, unsaturated fatty acid and DNA (Wagner et al. 2004). It causes nucleic acid modification by reacting with deoxyguanosine (Kasai 1997). It is also found to be highly responsible ROS which may trigger cell death (Krieger-Liszkay et al. 2008). $^1O_2$ can be quenched by nonenzymatic antioxidant or with D1 protein of photosystem II (Krieger-Liszkay 2005).

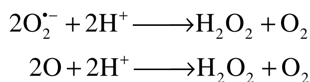
1.4.1.2 $O_2^{\bullet-}$

The oxygen radicals are highly reactive, producing hydrogen peroxides with enes and dienes

(Salim 1987). Moreover few particular amino acids such as histidine, methionine and tryptophan can be oxidised to $O_2^{\bullet-}$ (Knox and Dodge 1985), will cause lipid peroxidation in a cellular environment and lead to the disruption of cell membrane (Halliwell and Gutteridge 1989). By the process of enzyme or metal catalysed reactions a primary ROS $O_2^{\bullet-}$ is formed in the cell which in turn starts a chain of reactions to generate "secondary" ROS (Valko et al. 2005) depending upon the type of cell or cellular components. $O_2^{\bullet-}$ is a moderate reactive nucleophilic reactant species which is short-lived ROS having both oxidising and reducing properties (Halliwell 1997). It causes the oxidation of enzymes containing 4fe-4s clusters and reduces cytochrome *C* (McCord et al. 1977).

1.4.1.3 H_2O_2

It is formed when $O_2^{\bullet-}$ accepts one electron and two protons. Nonenzymatic or SOD-catalysed reaction can easily dismute to H_2O_2 (hydrogen peroxide)



$$2O_2^{\bullet-} + 2H^+ \longrightarrow H_2O_2 + O_2$$
$$2O + 2H^+ \longrightarrow H_2O_2 + O_2$$

H_2O_2 is produced easily in the cells under normal or stressful condition such as drought, chilling, UV irradiation, exposure to light, wounding and pathogen attacks. H_2O_2 is generally produced during electron transport chain of chloroplast, mitochondria, ER and plasma membrane or by β-oxidation of fatty acid and photorespiration. H_2O_2 is moderately reactive and long-lived molecule (Mittler and Zilinskas 1991). Besides other types of free radicals, H_2O_2 can easily cross the membrane and lead to the oxidative damage in the cell. It is beneficial as well as relatively stable than the molecules which are associated with H_2O_2 in the regulation of particular metabolic processes and activate the defence system in plants (Yan et al. 2007). High concentration of H_2O_2 can inactivate the enzymes involved at a specific level, and the enzymes lose 50 % of their activity (Dat et al. 2000). It also causes oxidation of protein enzymes and transcription factor and also triggers programmed cell death. It has been found that transgenic plants with increased levels of H_2O_2 due to the constituent overproduction of glucose oxidase or suppression of peroxisomal catalase were more resistant to pathogen accumulated SA and expressed PR genes and protein (Chammongpol et al. 1998). Pretreatment of maize seedling with H_2O_2, a SOD-generating compound that activates tolerance to chilling (Prasad et al. 1994). Plants regenerated from potato nodal explant treatment with H_2O_2 are found to be more resistant to temperature than the controlled plants (Lopez- Delgado et al. 1998). A recent study indicates that H_2O_2 promotes seed germination of various plants such as *Arabidopsis thaliana, Hordeum vulgare, Oryza sativa, Triticum aestivum* and *Helianthus annuus* (Yushi et al. 2013).

1.4.1.4 OH

OH is the most highly reactive molecule amongst all ROS. It has a single unpaired electron; thus, it can react with oxygen in triplet ground state. $^{\bullet}OH$ interacts with all biochemical molecules and causes extensive cellular damages such as lipid peroxidation, DNA and protein damage and membrane disruption (Foyer et al. 1997). As cells do not exhibit any defence system to abolish $^{\bullet}OH$ and thus its excess generation subsequently leads to apoptosis (Pinto et al. 2003). $^{\bullet}OH$ is produced under illumination, via Fenton reaction at the active site of the enzyme (RbcL) which results in its breakdown in chloroplast lysates (Luo et al. 2002). $^{\bullet}OH$ involves two essential reactions, viz. addition of $^{\bullet}OH$ to organic molecules or elimination of hydrogen atom from it. As $^{\bullet}OH$ are short-life period molecules having an extensive positive redox potential (close to +2V) of "free" $^{\bullet}OH$, the sites at which they react are nearer to the point at which they are formed (Elstner 1982). In this context, organic oxygen radicals such as alkoxy, peroxy, semiquinones, reduced hydrogen peroxide and hydrogen peroxide-electron donor complexes (crypto-OH), as well as metallo-oxygen complexes, have been described as the ultimately active species despite destructive-free $^{\bullet}OH$ (EF Elstner 1987).

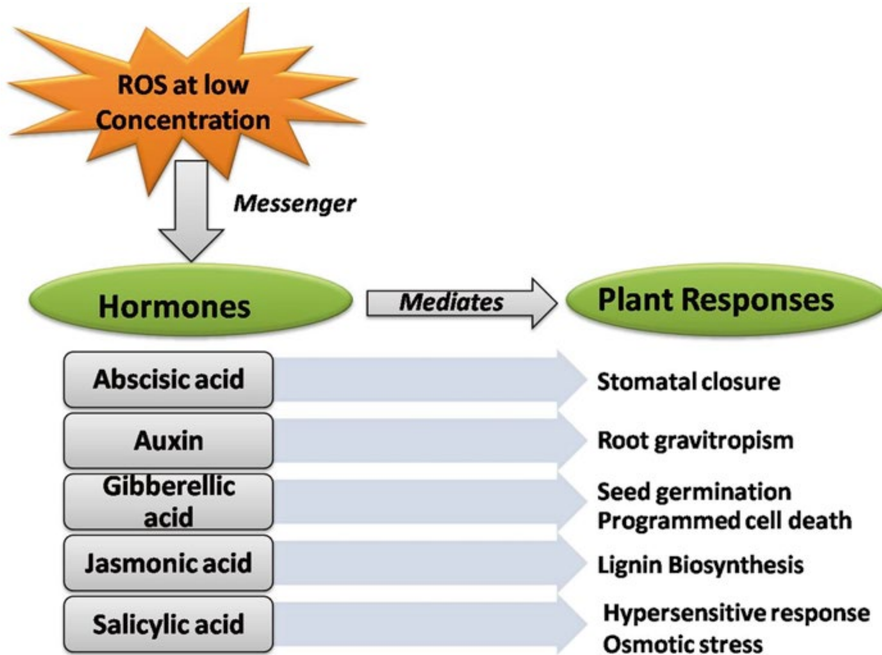


Fig. 1.6 Reactive oxygen species (*ROS*) as second messengers in several plant hormone responses

1.4.2 ROS as Second Messenger in Plants

ROS has been involved as a second messenger in intracellular signalling cascade as they activate various response in plants cells which includes tolerance to biotic and abiotic stresses (Miller et al. 2008), gravitropism (Jung et al. 2001), stomatal closure (Yan et al. 2007) and programmed cell death (PCD) (Mittler 2002). It has also been found that ROS mediates the hormonal response in plant as second messenger (Fig. 1.6). With the help of some redox-sensitive proteins, calcium mobilisation, protein phosphorylation and gene expression, the plant analyses transduce and allocate ROS signals into appropriate responses. ROS can be directly sensed by tyrosine phosphate signalling proteins (Xiong et al. 2002). Many component activities can be modulated by ROS and also interacts with other signalling molecules that regulate the response of downstream ROS by the pathway that forms a part of the signalling network (Neill et al. 2002). Miller and co-workers, using a mutant deficient in key ROS-scavenging enzymes, isolate a signalling pathway that is stimulated by the synthesis of ROS in the cells (Miller et al. 2008), and the important molecules implicated in this pathway are zinc finger proteins, WRKY transcription factors, that mediate the abiotic stresses which include temperature, salinity and osmotic stresses. ROS are referred to as the second messenger in the abscisic acid (ABA) transduction mechanism in guard cells (Yan et al. 2007). The plasma permeable channel reduces, which ultimately loss to mediate stomatal closure signal which is induced by ABA (Pel et al. 2000). It has been found that the constitutive increase of H_2O_2 is induced by ABA functions in stomatal closure (Jannat et al. 2011). The biosynthesis of drought-induced abscisic acid in plants by ROS plays an important role and suggests that they may be the signals through which the plant can "sense" drought conditions. The metals Cd^{2+} and Cu^{2+} induce activation of MAPK through ROS-generating system by using pharmacological inhibitors (Yeh et al. 2007). ROS can also function as second messenger in root gravitropism.

Joo et al. proposed that the mobilisation of auxin in plants by gravity stimulates generation of ROS to mediate gravitropism (Jung et al. 2001). Root gravitropism is inhibited by the scavenging activity of ROS by antioxidants (Jung et al. 2001). ROS is also involved in dormancy alleviation, and under normal conditions, gibberellic acid (GA) signalling and ROS content are lower in dormant barley grains under controlled condition. A pronounced effect has been found on GA signalling by exogenous H_2O_2 but does not have any effect on ABA biosynthesis and signalling and results in germination by activating a change in hormonal balance (Bahin et al. 2011). ROS have been found to play a critical role in programmed cell death in barley aleurone cells that are activated by GA. One of the studies has revealed that ROS are constituents of the hormonally mediated cell death mechanism in barley aleurone cells by observing that GA-treated aleurone protoplasts are less resistant to internally generated or externally applied H_2O than ABA-treated protoplasts (Bethke and Jones 2001). Increased generation of ROS is observed in the early onset of plant-pathogen communication and plays an essential signalling role in the pathogenesis of signal transduction regulators (Nanda et al. 2010). In HR, SA is thought to be potential ROS signalling molecules (Klessig et al. 2000). Expression of a salicylate hydroxylase (NahG) gene of transgenic *Arabidopsis* that is resistant to the increased osmotic stress may result from the reduced SA-stimulated production of ROS (Borsani et al. 2001). In response to wounding the ROS are found to act as second messenger for the activation of defence genes in tomato plants (Orozco-Cárdenas et al. 2001). In the leaves of tomato, ROS were produced close to the cell wall of vascular bundle cells in response to wounding and lead in the generation of H_2O_2 from wounding inducible polygalacturonase that acts as a second messenger for the activation of defence genes in mesophyll cells but not for signalling pathway genes in vascular bundle cells (Orozco-Cárdenas et al. 2001). Lignin plays a key role in mediating defence responses of plants to environmental changes. Through the interaction between jasmonic acid and ROS, a metabolic network that facilitates the plants to stimulate the accumulation of lignin in response to damage of cell wall has been recently characterised (Denness et al. 2011). ROS also plays an important role in heavy metal signal transduction pathway (Yan et al. 2007). ROS upregulates the genes implicated in osmotic stress signalling that include transcription of DREB2A and a histidine kinase (Desikan et al. 2001). It has been revealed that MAPK AtMPK6 can be stimulated by low temperature in *Arabidopsis* cell cultures and osmotic stress could also be activated by oxidative stress (Yuasa et al. 2001).

1.4.3 Adverse Effects of ROS

The production of ROS is a biochemical process due to oxidative metabolism of the cell. A serious imbalance between ROS formation and antioxidants levels causes oxidative stress. Oxidative stress is caused by the presence of low levels of antioxidant or by the increased production of ROS by environmental stresses such as salinity, drought, pathogen attacks and toxicity due to heavy metal (Halliwell 1997). Extensive formation or inadequate removal of ROS results in oxidative stress which may cause various metabolic malfunctions and extreme damage to biological macromolecules (Chopra and Wallace 1998). Oxidative stress leads to the production of extremely reactive oxygen species that are lethal to the cell, specifically the cell membrane in which these reactive molecules intercommunicate with lipid bilayer and generate lipid peroxides. The oxidative stress causes enzyme inhibition, damage to protein synthesis, DNA-strand breakage and lipid peroxidation (Fig. 1.7) that finally leads to apoptosis (Devasagayam et al. 1999).

1.4.3.1 Lipids

The oxidative deterioration of membrane lipid is called lipid peroxidation LPO (Horton and Fairhurst 1987). Lipid in biological systems undergoes autoxidation through a cascade of reactions involving three steps: initiation, propagation and termination. The first step involves

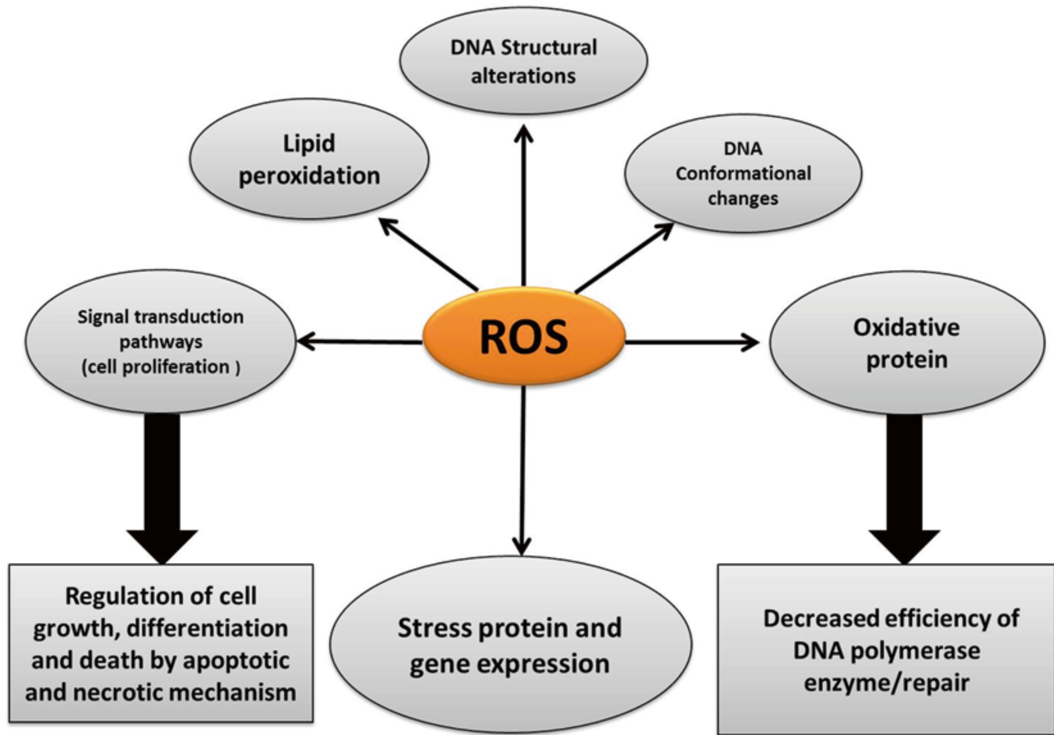


Fig. 1.7 Adverse effects of ROS

lipid peroxidation which is initiated by the reaction of an activated free radical such as singlet oxygen ($^1O_2$, $O_2^{\bullet-}$, or $^{\bullet}OH$) with a lipid substrate (LH) to produce extremely reactive carbon-centred lipid radical ($^{\bullet}L$). In the second step of LPO, molecular oxygen adds quickly to generate lipid peroxyl radical ($LOO^{\bullet}$). The $LOO^{\bullet}$ eliminates a hydrogen atom from another lipid molecule (LH), generating lipid hydroperoxide (LOOH) and another extremely reactive carbon-centred radical ($L^{\bullet}$) which then elongates the chain reaction, and the third step involves termination of lipid peroxidation that occurs through coupling of any two radicals to form non-radical products (NRP). NRP are stable but not able to propagate lipid peroxidation reactions (Porter et al. 1995). Transition metal ions such as copper and iron are essential in LPO (Fig. 1.8). Besides increasing the generation of initiating hydroxyl radicals, ferrous (Fe^{2+}) and ferric (Fe^{3+}) can catalyse the elongation of LPO chain by degrading LOOH. The resulting alkoxyl ($LO^{\bullet}$) and peroxyl ($LOO^{\bullet}$) radicals are able to induce new radical chains by interacting with additional lipid molecules.

Lipid peroxidation (LPO) of the cell membrane has been implicated in a number of physiological processes such as increased membrane rigidity, reduced cellular deformity and lipid fluidity in erythrocytes (Matkovics et al. 1998). When ROS formation crosses the equilibrium level in both cellular and organellar membranes, lipid peroxidation takes place spontaneously which results in affecting normal cellular functioning. Lipid peroxidation results in the generation of lipid free radicals that interacts with biomolecules and leads to the damage of proteins and DNA. It has been found that in plants under stressed condition, increased degradation of lipids takes place along with enhanced generation of ROS (Mishra and Dubey 2011). Malondialdehyde (MDA) one of the lipid oxidation by-products is responsible for the damage of cell membrane (Halliwell 1989). The polyunsaturated fatty acid present in phospholipids is very sensitive to ROS attack.

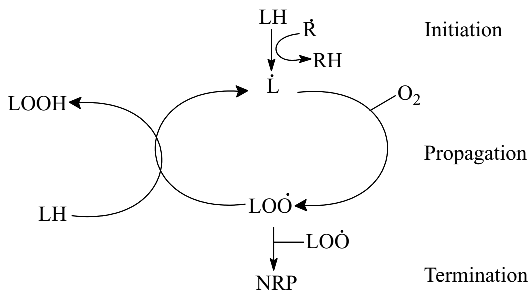


Fig. 1.8 Overview of lipid per-oxidation (Waldeck and Stocker 1996)

1.4.3.2 Proteins

There are direct and indirect effects of ROS on proteins which results in the alteration in various ways. Direct modification of protein activity takes place through nitrosylation, carboxylation, disulphide bond formation and glutathionylation, and the indirect modification takes place through conjugation with breakdown products of fatty acid peroxidation (Yamauchi et al. 2008). Extreme generation of ROS and modified electric charge elevates the sensitivity of proteins to proteolysis. Oxidative stress causes injury to tissues which generally contain high concentration of carbonylated proteins, commonly used markers of protein oxidation (Moller and Kristensen 2004). The plants under stress result in enhanced modification of proteins (Tanou et al. 2009). Oxidation of iron-sulphur centres by $O_2^{\bullet-}$ is invariable and results in the suppression of enzyme (Gardner and Fridovich 1991). Due to metal treatment, oxidised proteins were more effectively decomposed and the proteolytic activity increased by 20 % (Romero-Puertas et al. 2002). Several studies have described that more increase in damage leads to the inactivation of protein enzymes (Grune et al. 1997).

1.4.3.3 DNA

ROS contributes as a major source of DNA damage (Imlay and Linn 1988). DNA is the genetic material of the cell, and damage to the DNA can lead to the malfunctioning of many important proteins. The oxidative damage caused by ROS particularly hits nuclear, mitochondrial and chloroplastic DNA. The attack of ROS on DNA results in the strand lesions, sugar oxidation, elimination of nucleotides and DNA protein interlinks. Moreover alteration in the nucleotides on both the strands results in subsequent mutations. Environmental stresses such as salinity (Liu et al. 2000) and metal toxicity (Meriga et al. 2004) lead to the enhanced degradation of DNA in plants. Both the sugar and base components of DNA are more sensitive to oxidation by ROS. Mutagenic alteration can be caused by the attack of ROS on DNA (Fink et al. 1997). ROS attack to DNA sugar leads to strand breakage (Evans et al. 2004). Because of the absence of shielding proteins, histones and near location of ROS, nuclear DNA was less sensitive to oxidative stress than mitochondrial and chloroplastic DNA (Richter 1992). Extensive alterations induced by ROS result in the unaltered changes of DNA with extreme effects for the cell, despite the repairing system is already present for the destructed DNA.

1.4.4 Defence Mechanism in Plants

Oxidative stress generated during pathophysiological conditions may be effectively neutralised by enhancing the cellular defences in the form of antioxidants (Devasagayam et al. 1999). Antioxidants are a group of substances, when present at low levels, in relation to oxidisable substrates, considerably suppress or delay oxidative processes, while often being oxidised themselves. The content of protective antioxidants present under normal metabolic conditions is sufficient only to combat with physiological rate of free radical production. It is obvious, therefore, that any additional burden of free radicals from environment or generated within the body can disrupt the free radical (pro-oxidant) and anti-free radical (antioxidant) balance (Davies 1995). Depending upon their mechanisms of action, antioxidants act in two different ways as radical chain breaking antioxidants and preventive antioxidants. Several compartments of antioxidative defence mechanism that have been implicated in ROS quenching activity have been altered, overexpressed or downregulated that adds to the current information and understanding the role of

the antioxidant defence mechanisms. The defence mechanism of plant system comprises of enzymatic and nonenzymatic defence systems shown as follows:

1.4.4.1 Nonenzymatic Defence System

The nonenzymatic defence system includes the major cellular redox buffer ascorbate (ASA) and glutathione (GSH) as well as tocopherols, carotenoids and phenolic compounds. They have a crucial role in defence and as enzyme co-factor, as they interact with many cellular components; these antioxidants influence the plant growth and development by regulating process from mitosis and cell elongation to senescence and apoptosis (De Pinto and De Gara 2004). It has been found that the mutants with decreased nonenzymatic antioxidant content are hypersensitive to stress (Semchuk et al. 2009).

Ascorbate (ASA)

ASA is the most commonly found, low molecular weight antioxidant that plays an essential role against oxidative damage caused by the increased production of ROS. It has the capability to donate electrons in a wide variety of enzymatic and nonenzymatic reactions. It has also played a key role in plant growth, differentiation and metabolism. Ascorbate exists in diverse plant cell types, organelle and apoplast (Smirnoff et al. 2004). Ninety percent of ASA is localised in cytoplasm but majority is present in apoplast. It has been found that apoplastic ASA shows basal defence against external oxidants (Barnes et al. 2002). ASA also functions as a co-factor of violaxanthin de-epoxidase when present in reduced state in chloroplast thus sustaining the dispersion of extensive excitation energy and also protects macromolecules from oxidative damage (Smirnoff 2000). It directly reacts with $O_2^{\bullet-}$, H_2O_2 and generating α-tocopherols and preserves the activity of enzymes (Noctor and Foyer 1998) and also provides protection to membranes. ASA plays a crucial function by eliminating H_2O_2 through AsA-GSH cycle (Pinto et al. 2003). It has been observed that the content of ASA depends on the balance between the rate and capacity of ASA accumulation and output of antioxidant demand under stress conditions (Chaves et al. 2002). Overexpressing of enzymes that have been implicated in ASA accumulation results in abiotic stress resistance in plants. In tomato plants, overexpression of two members of the GME gene family leads to the elevated biosynthesis of ascorbate and resistance to abiotic stress (Zhang et al. 2011). A recent study has observed that the overexpression of strawberry D-galacturonic acid reductase in potato plants results in the biosynthesis of ASA and increase abiotic stress resistance (Hemavathi et al. 2009). Similarly stress tolerance in Arabidopsis has been shown by increased content of ASA (Wang et al. 2010). The Vtc-1 mutant has shown high susceptibility to supplementary UV-B treatment than wild type (Gao and Zhang 2008) as it lacks the function of GDP-mannose pyrophosphorylase (Wheeler et al. 1998).

Glutathione

γ-Glutamyl-cysteinyl-glycine (GSH) is a low molecular weight nonprotein thiol playing an essential role in combating oxidative stress mediated by ROS. It is virtually present in cytosol, chloroplast of ER, vacuoles and mitochondria (Foyer and Noctor 2003). Cellular redox state was maintained by balancing the GSH and glutathione disulphide (GSSH). GSH plays an important role due to its reducing power in many biological processes, including signalling, conjugation of metabolites, enzymatic regulation, accumulation of proteins and nucleic acid and the expression of stress responsive genes (Foyer et al. 1997). It acts as a scavenger because it chemically reacts with $O_2^{\bullet-}$, $^{\bullet}OH$ and H_2O_2. GSH can protect proteins, lipids and DNA either by the generation of adducts or by functioning as proton donor in the presence of ROS yielding GSSH (Asada 1994). Plants under various stresses like salinity (Hefny and Abdel-Kader 2009), chilling (Radyuk et al. 2009) and metal toxicity (Mishra and Dubey 2011) have been found that altered ratios of GSH/GSSH are present. In the GSH biosynthesis, the overexpression of enzyme glutathione synthetase is unable to show an

impact on GSH formation and observed that it was incapable to induce ozone resistance (Strohm et al. 1999) and resistance to photo inhibition (Foyer et al. 1995) in hybrid proteins. Overexpression of γ-ECS results in less susceptibility towards cadmium stress in Indian mustard (Zhu et al. 1999) and increased resistance towards chloroacetanilide herbicide in polar plants (Gullner et al. 2001). With the high level of reduced glutathione in transgenic potato, it results in higher protection against oxidative damage stimulated by several abiotic stresses (Eltayeb et al. 2010).

Tocopherols

Different types of α-, β-, γ- and δ-tocopherols belong to a group of lipophilic antioxidants implicated in oxygen free radical, lipid peroxyl radicals and $^1O_2$-scavenging activity (Diplock et al. 1989). Amongst tocopherols α-tocopherols contain the highest antioxidant activity (Kamal-Eldin and Appelqvist 1996). The main function of tocopherols is to protect lipids and other membrane compartments and hence defend the structure and function of PSII by metabolically scavenging and biochemically reacting with chloroplast O_2 (Ivanov and Khorobrykh 2003). Accumulation of α-tocopherols in different plant species found to activate resistance to chilling, water deficit and salinity (Bafeel and Ibrahim 2008). One of the studies has described that expression level of genes encoding enzyme of AsA-GSH cycle were stimulated, viz. APX, DHAR and MDHAR (Li et al. 2010a). Transgenic rice plant with Os-VTE1 RNA interference show high susceptibility to salt stresses, while as in transgenic plants overexpressing Os-VTE1 results in extreme resistance to salt stress (Ouyang et al. 2011).

Carotenoids

Carotenoids also represent a group of lipophilic antioxidant that is able to remove several types of ROS (Young 1991). Carotenoids as an antioxidant quench $^1O_2$ to suppress oxidative stress. Carotenoids also act as precursors to signalling molecules that controls plant development and abiotic/biotic stress response (Li et al. 2008). It has been found that extreme carotenoids amount shows good adjustment of sugar cane plants under salinity stress (Gomathi and Rakkiyapan 2011).

Phenolic Compounds

Phenolic compounds are varied secondary metabolites (flavonoids, tannis, hydroxylcinnamate esters and lignin) which exhibit antioxidant activity and found in plant tissues (Grace and logan 2000). Polyphenols contain an aromatic ring with ^-OH or OCH_3 substituent that acts synergistically which contributes to their metabolic functions including antioxidant activity. Polyphenols also change lipid packing order and reduce membrane permeability (Arora et al. 2000). In response to different stresses, there are evidences for the activation of phenolic metabolism in plants (Michalak 2006). It has been observed that ROS might involve in the biosynthesis of total phenolic compound in dark-grown lentil roots (Janas et al. 2009); with increased amount of flavonoid concentration, the transgenic potato plant showed higher antioxidant activity (Lukaszewicz et al. 2004).

Proline

α-Amino acid is an antioxidant and potential inhibitor of programmed cell death. It has been suggested that free proline acts as osmoprotectant, a protein stabiliser, a metal chelator, an inhibitor of lipid peroxidation and $OH^\bullet$ and $^1O_2$ scavenger. Increased proline accumulation appears especially during salt, drought and metal stresses (Trovato et al. 2008). Thus, proline is not only an essential signalling molecule but also an efficient ROS scavenger. It has been found that the important role of proline is in potentiating pentose-phosphatase pathway activity as essential compartment of antioxidative defence system (Hare and Cress 1997).

1.4.4.2 Enzymatic Defence System

The enzymatic components of the antioxidant mechanism that includes various antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GR) (Noctor and Foyer 1998) (Fig. 1.9).

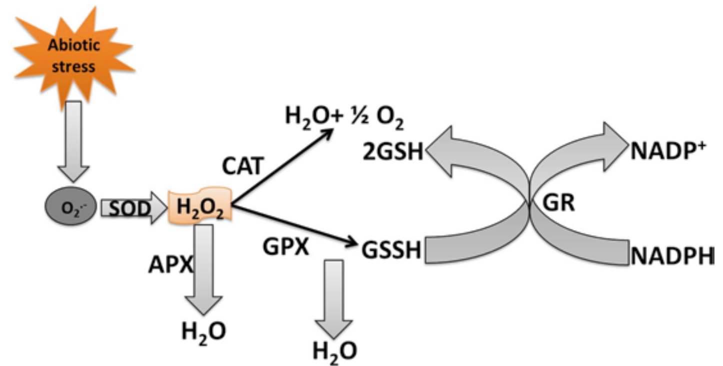


Fig. 1.9 ROS and enzymatic antioxidant defense mechanisms

SOD

Superoxide anion is a reduced form of molecular oxygen evolving by receiving an electron. Superoxide anion an initial free radical plays an essential role in the generation of reactive oxygen species in biological systems (Steif 2003). SOD converts superoxide anion into hydrogen peroxide and oxygen. There are three types of SOD: a Mn-containing SOD, a Cu/Zn-dependent SOD and extracellular SOD (Racchi et al. 2001). SOD plays a critical role in defence mechanism against oxidative stress in all aerobic organisms (Scandalios 1993). Mn-SOD is localised in chloroplast (Jackson et al. 1978), and Cu/Zn SOD is found in cytosol, chloroplast, peroxisomes and mitochondria (Del Rio et al. 1998). Due to drought and metal toxicity, the activity of SOD has been found to enhance in the plants (Mishra et al. 2011) and has been found often mutually related with elevated resistance of plants to environmental stresses. SOD can be used as an indirect selection criterion for screening drought-resistant plant material (Zaefyzadeh et al. 2009). Increased oxidative stress resistance in plants has been reported by the overproduction of SOD (Gupta et al. 1993).

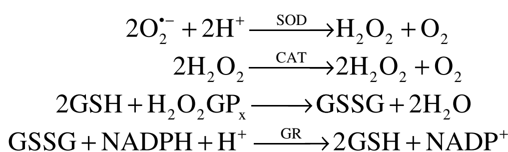
Catalase

Catalase, a tetrameric enzyme, is localised mainly in peroxisomes, while reduced levels also exist in mitochondria and cytosol. The human erythrocytes are rich in catalase (Aebi 1983). Catalase is involved in cellular detoxification and converts H_2O_2 to water and oxygen. Catalase protects cells from H_2O_2 production within them. Although catalase is not crucial for some cell types under normal conditions, it plays a critical role in developing resistance to oxidative stress in adaptive response of cells (Grazioli et al. 1998). Amongst antioxidant enzymes, catalase (CAT) was the first enzyme to be characterised. CAT has extreme specificity for H_2O_2 but less activity against organic peroxides. CATs are rare enzymes as they do not need cellular reducing equivalent, and plants exhibit various types of H_2O_2 decomposing enzymes. CAT exhibits less affinity for H_2O_2 than APX but higher turnover rate. During photorespiratory oxidation, CAT scavenges H_2O_2 further β-oxidation of fatty acid and other enzymes such as XOD coupled SOD (Corpas et al. 2008). One report proposed grouping of CATs based on the expression profile of tobacco genes. Class I CATs are mediated by light and expressed in photosynthetic tissues, Class II CATs are expressed in vascular tissues, whereas Class III CATs are expressed in seeds (Willekens et al. 1995). H_2O_2 is degraded by CAT in an energy efficient manner (Mallick and Mohn 2000). It has been found that environmental stress either enhanced or reduced activity of CAT depending on the magnitude of strength and type of stress (Moussa and Abdel-Aziz 2008). Stress analysis has described that enhanced sensitivity of CAT-deficient plants to paraquat salt and ozone but not chilling (Willekens et al. 1997). CAT is crucial for regulating the redox balance during oxidative stress that is indicated by CAT activity which shows biosynthesis of GSSH and a 4-fold reduction in ASA in transgenic tobacco plant having 10 % wild type (Willekens et al.1997). A CAT gene

isolated from *Brassica juncea* introduced and overexpressed in tobacco plants increased its resistance to cd-induced oxidative stress (Guan et al. 2009). Catalase was found to be receptor and when SA binds it inactivates CAT. Catalase inactivation results in H_2O_2 synthesis, which was shown to act as a secondary messenger to stimulate pathogen-related (PR) gene (Chen et al. 1993). By using the sense and antisense technology in transgenic Nicotiana lines (CATIAS), only 10 % of the residual catalase activity was retained (Chamnongpol et al. 1996). In *Arabidopsis thaliana*, *Nicotiana plumbaginifolia*, *Oryza sativa* and *Zea mays,* cDNAs that code for three different classes have been isolated (Frugoli et al. 1998). *N. plumbaginifolia* contains three active catalase-encoding genes (cat1, cat2, cat3); two of which are expressed in mature leaves (Willekens et al. 1994). Increased susceptibility against ozone and salt stress of the CATIAS plants indicates that catalase activity is crucial for cellular defence against the environmental stress that cause H_2O_2 generation from photorespiration (Willekens et al. 1997). It has been found that in *Arabidopsis* mutants, phytoalexin biosynthesis in parsley cell suspension and lesion formation is particularly activated by SOD not by H_2O_2 (Jabs et al. 1996). There is spontaneous reduction in CAT1, CAT2 transcript and protein levels and decrease in total catalase action which is correlated with the accumulation of H_2O_2 in the tobacco cells that sustains HR upon infiltration with fungal elicitors (Dorey et al. 1998).

Glutathione Reductase

Glutathione peroxidase is an essential hydrogen peroxide-removing enzyme present in the membranes. Glutathione disulfide reductase, a flavoprotein, allows the transformation of oxidised glutathione (GSSG) to reduced glutathione (GSH) by oxidation of NADPH to $NADP^+$ (Papas 1999).



$$2O_2^{\bullet-} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$
$$2H_2O_2 \xrightarrow{CAT} 2H_2O_2 + O_2$$
$$2GSH + H_2O_2GP_x \longrightarrow GSSG + 2H_2O$$
$$GSSG + NADPH + H^+ \xrightarrow{GR} 2GSH + NADP^+$$

Superoxide anion itself is not a strong oxidant, but it reacts with protons in water solution to form hydrogen peroxide, which then serves as substrate for the production of highly reactive species such as hydroxyl radicals and singlet oxygen (Steif 2003). As an antioxidant GR plays an essential role in enzymatic as well as nonenzymatic redox cycle in which oxidation of GSH to GSSH takes place. GR represents a group of flavoenzymes and exhibits an important disulphide group (Ghisla and Massey 1989). Two steps involved in catalytic mechanism of GR, viz. (1) the moiety is reduced by NADPH and (2) reduction of GSSH via thiol disulphide interchange reaction (Ghisla and Massey 1989). It is present in chloroplast, cytosol, mitochondria and peroxisomes, and 80 % of GR activity is found to be present in photosynthetic tissues and chloroplast isoforms (Edwards et al. 1990). Environmental stresses increase the activity of GR (Maheshwari and Dubey 2009), and it has been found that antisense-mediated depletion of tomato chloroplast GR has been shown to enhance susceptibility to chilling (Shu et al. 2011). Extreme amount of foliar ASA and increased resistance to stress have been shown by overexpression of the GR in *N. tobacco* and *populous* plant (Foyer et al. 1995). As the ROS detoxification system is very complex, it has been observed that overexpressing of antioxidant defence system may not change the capability of the entire pathway (Lee et al. 2009). Several studies have shown that in transgenic plants the overexpression of combination of antioxidant enzymes has synergistic effect on stress resistance (Kwon et al. 2002). Overexpression of genes of SOD and APX in chloroplast, (Kwak et al. 2009) SOD and CAT in cytosol (Tseng et al. 2008) and SOD and GR in cytosol (Aono et al. 1995) has been shown to result in increased resistance to different environmental stresses. It is important to note that much importance is now given to generate transgenic plants with overexpression of different types of antioxidants for achieving resistance in order to combat environmental stresses.

Guaiacol Peroxidase

Guaiacol peroxidase, a haeme-containing protein, is commonly found in microbes, plants and animals and is mostly oxidised by guaiacol and

pyrogallol electron donors at the cost of hydrogen peroxide. It has been found that guaiacol (anionic) peroxidase exhibits about 90 % of peroxidase activity in plants (Foyer et al. 1994). This haeme-containing protein is found to be involved in the accumulation of lignin and suppression in the formation of indole-3-acetic acid (IAA) and provides defence system against pathogen that inhales H_2O_2 (Kobayashi et al. 1996). It has been revealed that several isoenzymes of GPX are found to be present in tissues that are mobilised in vacuoles, the cell wall and the cytosol of the plant (Asada 1992). It has been found that GPX plays an efficient role by scavenging of O_2 and peroxyl radical under stressful conditions and commonly termed as "stress" enzyme (Vangronsveld and Clijsters 1994). In response to biotic and abiotic stresses, there is a rapid stimulation of GPX activity in plants (Moussa and Abdel-Aziz 2008). One of the studies carried out by Radotic et al. (2000) suggested that during oxidative stresses in response to metal toxicity, GPX can be used as biomarker for not so lethal toxicity of metals in plants by correlating increased activity of GPX. Current studies have described that GPX exhibits an essential role against salt resistance of safflower plants which is increased by increasing the GPX activity in the plants (Tayefi-Nasrabadi et al. 2011).

1.4.4.3 Enzymes of Ascorbate-Glutathione Cycle

In the cell, there is change in the ratio of AsA to DHA and GSH to GSSG which is critical state for the cell to identify oxidative stress and functions in an appropriate manner. The AsA-GSH cycle also termed as Halliwell-Asada pathway is the recycling pathway of AsA and GSH production which also eliminates H_2O_2 and is present in four different subcellular locations, including the cytosol, chloroplast, mitochondria and peroxisomes (Jiménez et al. 1997). The AsA-GSH pathway involves extensive redox reactions of AsA, GSH and NADPH catalysed by the enzymes APX, MDHAR, DHAR and GR. During environmental stresses AsA-GSH pathway plays an essential role in overcoming oxidative stress (Sharma and Dubey 2005).

Ascorbate Peroxidase

Ascorbate peroxidase (APX) is a main component of AsA-GSH cycle and plays an important role in mediating internal responses of ROS levels. It belongs to a class-I superfamily of haeme peroxidase (Welinder 1992) and is mediated by redox signals and H_2O_2 (Patterson and Poulos 1995). The reaction involves the use of two molecules of ASA to reduce H_2O_2 to water with a simultaneous generation of two molecules of MDHA. APX exists in cytosol, apoplast and other organelles playing an important role in scavenging H_2O_2 within the organelle, whereas cytosolic APX detoxifies H_2O_2 generated in the cytosol, apoplast or that diffused from organelles (Mittler and Zilinskas 1992). During stressful conditions CAT makes APX less effective in mediating scavenging activity to eliminate H_2O_2, whereas isoforms of APX have much higher affinity for H_2O_2 and are known as one of the most commonly found antioxidant enzyme in the plant cell (Wang et al. 1999). Several studies have observed that in response to abiotic stresses such as drought, salinity, chilling, metal toxicity and UV irradiations, the APX activity is accelerated considerably (Hefny and Abdel-Kader 2009). It has been found that in transgenic tomato plants, overexpression of a cytosolic APX gene isolated from pea decreases the oxidative injury induced by chilling and salt stress (Wang et al. 2005). Similarly in *Nicotiana tobaccum* or *Arabidopsis thaliana,* overexpression of tAPX gene increased resistance to oxidative stress.

Monodehydroascorbate Reductase

In plants, the monodehydroascorbate reductase (MDHAR) is an enzymatic component of the glutathione-ascorbate cycle that is one of the major antioxidant systems of plant cells for the protection against the damages by reactive oxygen species (ROS). The MDHAR activity has been observed in several cell compartments, such as chloroplasts, cytosol, mitochondria, glyoxysomes and leaf peroxisomes, and various isoenzymes of MDHAR have been found to exit in various cellular compartments like chloroplasts (Hossain et al. 1984). Various studies have described that during environmental stresses the

activity of MDHAR is enhanced in plants (Maheshwari and Dubey 2009). It has been found that in tobacco, overexpression of *Arabidopsis* MDHAR gene results in the increased resistance to salt and polyethylene glycol stresses (Eltayeb et al. 2007) and similarly in transgenic *Arabidopsis* overexpression of tomato chloroplastic MDHAR increased its resistance to temperature and methyl viologen-mediated oxidative stresses (Li et al. 2010a, b).

Dehydroascorbate Reductase

Dehydroascorbate reductase involves in the stimulation of the symplastic and apoplastic ascorbate pool size and redox state of the cell. The reduction of dehydroascorbate to ascorbate is catalysed by DHAR at the expense of glutathione in the reaction. Thus, dehydroascorbate reductase catalyses the regeneration of ascorbate from its oxidised state and serves as an important regulator of ascorbate recycling. In plants overexpression of DHAR has been found that guard cells contain lower levels of H_2O_2 and decreased responsiveness to H_2O_2 or abscisic acid signalling, which results in higher stomatal opening (Chen and Gallie 2005). On the contrary, suppression of DHAR expression in guard cells results in higher levels of H_2O_2 and increased stomatal closure under normal growth conditions or following water deficit. It has been found that enhanced expression of DHAR results in the increased resistance to ozone in plants, while decreased expression of DHAR in plants results in the reduced rate of CO_2 assimilation with less growth and reduced biomass accumulation (Chen and Gallie 2005). One of the recent studies shows that overexpression of cytosolic Arabidopsis AtDHAR1 results in the enhanced resistance to herbicide, drought and salt stresses in transgenic potato plants (Eltayeb et al. 2011).

In addition to the above discussion, NO is one of the reactive oxygen species that have diverse functions in plant physiology. NO may be implicated in some of the defence responses mediated by the reactive oxygen species (ROS) such as defence gene activation, the hypersensitive cell death and phytoalexin biosynthesis (Durner et al. 1998). Nitric oxide (NO) is a ubiquitous signal molecule that has been implicated in a multiplex of plant responses to environmental stress. In the current years, the regulating role of NO on heavy metal toxicity in plants is realised increasingly, but knowledge of NO in alleviating aluminium (Al) toxicity is quite limited. There are many evidences that describe the role of NO in increasing Al toxicity in plants via activating defence mechanism to eliminate reactive oxygen species (Huyi et al. 2012). It has been suggested that ROS is not always sufficient to mediate a strong disease resistance response in plants, and their combination with NO can act synergistically to activate a stronger response. Many studies have reported the isolation of the enzymes that catalase NO synthesis and its involvement in many process includes its signalling functions at the molecular level (Lamotte et al. 2004). However, much information has been gaining in understanding the role of NO in plants over the past decade. NO functions in many metabolic processes that include generation, root growth, stomatal closing and adaptive responses to various environmental stresses (Delledonne 2005) in plants. Let us briefly discuss the role of NO in plant signal transduction and its function together with ROS in plant immunity.

1.5 Role of Nitric Oxide (NO) in Plant Signal Transduction

Nitric oxide (NO) is an inducible molecule or free radical reactive gas which has been found to be present in animals and other biological systems that play an essential role in various metabolic processes (Schmidt and Walter 1994). NO is a small molecule, having short half-life period, absence of charge and high diffusivity that would serve as an ideal inter- and intracellular signalling molecule in plant defence mechanisms. Many experiments suggest that NO is not generated only from nitrite but can be also produced from L-arginine (Corpas et al. 2006). Understanding the pathway, mechanism underlying NO synthesis and signalling activities in plants is still rudimentary. However, many studies have shown the essential function of NO as a

signalling molecule in plant systems. Nitric oxide (NO) is a reactive radical molecule and during plant signalling it has been synthesised in response to biotic and abiotic stresses. During life cycle of plants, it has been described that many essential developmental processes that can be mediated by this inducible molecule which includes plant growth and development, de-etiolation and gravitropism response are well defined. Besides the existing information about NO, the pathway of NO signalling is not yet fully elucidated, but there are evidences that explained the importance of NO as a stimulator of plant growth and stress is rising significantly. It has also been found that NO plays a crucial role in upregulating antioxidant defence system that contributes to increased resistance against high-temperature-induced oxidative damage in wheat (Bavita et al. 2012). In tomato plants it has been found that the primary root (PR) growth is lowered by NO, while lateral root (LR) development is accelerated (Correa-Aragunde et al. 2004). A study showed that NO inhibits the growth of roots and has been utilised as a morphological tool for screening of hypersensitive mutants of NO (He et al. 2004). By using this property of NO, it became possible to isolate NO-overproducer mutant nox1 and also found that CUE1 underexpressed as the mutant gene. It has been found that differentiation of NO takes place in a dose-dependent manner and also suggested the growth is inhibited by endogenous application of the extreme levels of NO, while the low levels promote it (He et al. 2004). Similar results have been found in plant gravitropic response in which addition of high level of NO on the upper side promoted elongation, while low levels of NO on the lower sides suppress it, thus effecting gravitropic bending (Hu et al. 2005). In *Arabidopsis* by using different analysis approach, it has been found that NO operates during onset of plant growth, but it also has been reported that PR growth of wild-type *Arabidopsis* seedlings is suppressed by NO (Fernandez-Marcos et al. 2011). One of the recent studies has observed that in *Euphorbia pekinensis* suspension cells, the endophytic fungal elicitor stimulates elevation in the amount of NO and generation of SA which in turn increases the biosynthesis of isoeuphpekinensin and also describes that ROS are not involved in the endophytic fungus-host interaction signalling pathway (Fu-Kang et al. 2012). One of the studies has also revealed that in *Catharanthus roseus* cells, the extracellular application of NO through its donor sodium nitroprusside (SNP) results in the generation of catharanthine (Xu et al. 2005). Mao-Jun et al. (2005) studied the impact of NO scavenger 2- to 4-carboxyphenyl-4,4, 5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPITO), nitric oxide synthase inhibitor S, S′-1, 3-phenylene-bis (1,2-ethanediyl)-bis-isothiourea and inhibitors of the octadecanoid pathway on elicitor-induced NO generation, JA biosynthesis and hypericin production. The results revealed a causal relationship exists between elicitor-induced NO production, JA biosynthesis and hypericin generation in *H. perforatum* cells and indicates a sequence of signalling events from NO to hypericin production within which NO mediates the elicitor-induced hypericin biosynthesis at least partially via a JA-dependent signalling pathway. The constitutive part of common signal transduction in plant defence mechanism contains JA and its methyl ester methyl jasmonate (MeJA) that are well-defined signalling molecules (Mueller et al. 1993). Many studies have been carried out and demonstrate that when the plant tissues and cells are subjected to external treatment of JA or MeJA, they have been found to imitate the impact of wounding and elicitor to stimulate the corresponding responses like accumulation of secondary metabolites and proteinase inhibitor enzymes (Gundlach et al. 1992) and rapid production of H_2O_2. One of the recent studies suggested that NO plays a crucial role in few JA-stimulated or JA-mediated defence mechanisms, viz. the inhibition of wounding-induced H_2O_2 production and proteinase inhibitor synthesis in tomato leaves (Orozco-Cárdenas and Ryan 2002) and MeJA-induced accumulation of H_2O_2 and malondialdehyde (MDA) in rice leaves (Hung and Kao 2004). Jian and Jian (2005) found a similar result in *Taxus chinensis* cell cultures in which nitric oxide (NO) plays an active role in MeJA-mediated plant defence responses and secondary metabolism. Similarly, it has been found

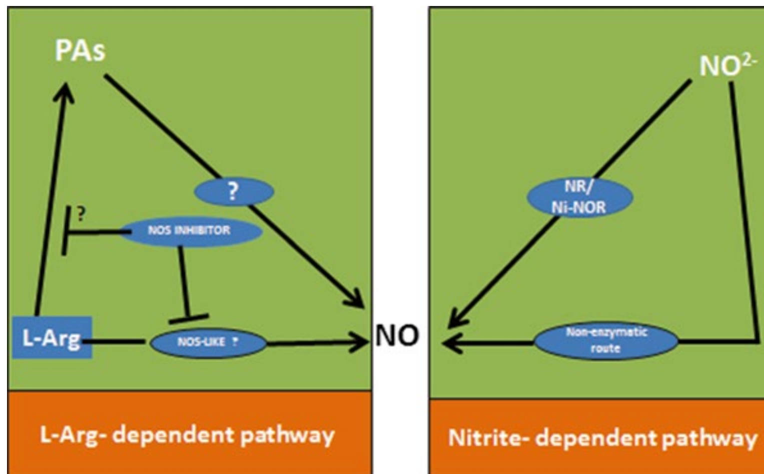


Fig. 1.10 Nitric oxide (*NO*) synthesis in plants: It comprises of two pathways L-Arg dependent pathway that involved NOS like enzyme and Nitrite-dependent pathway that involves NR, Ni- NOR and non-enzymatic pathway for the production of NO (Angelique Besson-Bard et al. 2008)

that in *Arabidopsis thaliana* JA and wounding activates a strong NO burst (Huang et al. 2004).

1.5.1 Pathways

Recent information known about NO signalling in plants revealed that there exists two different enzymatic pathways for the NO production in plants, viz. a nitrate-/nitrite-dependent pathway and an L-Arg-dependent pathway (Fig. 1.10). The enzyme involved in the first pathway is a cytosolic nitrate reductase NR (Yamasaki and Sakihama 2000) and a root-specific plasma membrane nitrite-NO reductase (Ni-NOR) (Stohr and stremlau 2006). The in vitro production of NO was catalysed by NR and uses NAD(P)H as an electron donor through one electron-nitrite reduction (Stohr et al. 2001). In vivo, the basic generation of NO levels in the leaves and roots of various plant species is induced by NR (Vanin et al. 2004), and the process is regulated by the phosphorylation state of enzyme (Lea et al. 2004). It has been found that in ABA-signalling of guard cells NR- induces NO synthesis is the main step; however by observing the transduction mechanism in *Arabidopsis thaliana* nia1, nia2 NR-deficient mutants ABA do not lead to the generation of NO during stomatal closure (Bright et al. 2006). NO formation from nitrite, Ni-NOR, is involved and formation of NO takes place extensively in roots. There is co-ordination between the Ni-NOR and the plasma membrane-bound NR that reduces nitrate to nitrite (Stohr et al. 2001). It has been observed that Ni-NOR is implicated in many physiological processes particularly related to root such as development, response to anoxia and root symbiosis (Stohr and Stremlau 2006), but the actual identity of Ni-NOR is not clear. Although many experiments have been carried out to elucidate the actual process involved and finally scientists have developed a nitrite-dependent mechanism for NO synthesis. These include the following:

(a) At the acidic pH in the apoplasm, there is reduction of nitrite to NO (Bethke et al. 2004).
(b) And production of NO in mitochondria due to reduction of nitrite to NO (Planchet et al. 2005).

However there exists no homology of animal NOS in the Arabidopsis genome (Arabidopsis genome initiative 2000). It has been suggested that NOS-like enzyme may be present in plant tissues and purified organelles (Tian et al. 2007). NO synthesis in plants can be suppressed by the use of mammalian NOS inhibitor and also cell suspension exposed to hormones (Zottini et al. 2007), derived elicitors or pathogen (Vandelle

et al. 2006) and salt stress (Zhao et al. 2007). Recent studies have described that a protein which is involved in the accumulation of NO in snail Helix pomatia shows sequence resemblance to the enzyme involves in the reactions of *Arabidopsis* results in the cloning of AtNOS1 gene which encodes mitochondrial enzyme in *Arabidopsis thaliana* (Guo and Crawford 2005). A study revealed that the AtNOS1 enzyme plays a critical role in floral transition (He et al. 2004) and in the signalling pathway of ABA that is the main source of NO (Guo et al. 2003) and lipopolysaccharide (LPS) (Zeidler et al. 2004). However it has been found that in specific environmental conditions such as in response to ABA or LPS, the mutant AtNOS1 shows the presence of reduced levels of NO, and it was recently questioned that how the mutant AtNOS1 exhibited the NOS activity (Zemojtel et al. 2006). It has been currently described that in the synthesis pathway of NO, there is an active involvement of an enzyme that is not known but has been found to play a role in the uninterrupted transformation of polyamines to NO. Recently a specific analysis has been carried out on the amount of amino acid in the leaves of *Arabidopsis* nia1, nia2 NR-deficient mutant grown, and it has been observed that a decrease of tenfold level of L-Arg is observed in presence of ammonia. The above observation suggests that there is an insufficient amount of endogenous substrate present in plants lacking nitrate or NR which is not able to generate NO either from the nitrate/nitrite-dependent pathway or from the L-Arg-dependent pathway.

1.5.2 NO Signalling in Plants

The metal nitrosylation, S-nitrosylation and tyrosine nitration play an important role in NO signalling in plants (Fig. 1.11). Recently it has been described that NO forms complex with metal-containing proteins in plants. In the recent years more focus has been given to clearly elucidate the reaction between NO and haemoglobin. Plants contain three main types of haemoglobin (Hb), viz. symbiotic Hb found in nitrogen-fixing root nodules of leguminous plants (Mathieu et al. 1998), non-symbiotic consists of two classes of Hb both containing extreme and reduced affinity for oxygen and truncated Hb (Dordas et al. 2004). It has been recently found that the root nodules of soya bean, cowpea and alfalfa contain $Lbfe^{II}$ NO and also described the presence of different forms of LB generated in vivo such as oxy Lb and ferryl Lb which are well defined for their involvement in the quenching activity of NO and peroxynitrite ($ONOO^-$) (Herold and Puppo 2005) in vitro. Similarly studies have shown that the different plant species containing class-I Hb catalyse the reaction involving NAD(P)H-dependent transformation of NO to nitrate in vitro (Seregelyes et al. 2004). Perazzolli et al. (2004) have described that *Arabidopsis* class-I Hb is also mediated via S-nitrosylation. Mutagenesis experiment recently has shown that this regulation might not be crucial for quenching of NO (Igamberdiev et al. 2006). One of the recent hypothesis supports that functional interaction between NO and class-I Hb by the use of both defective and overexpressing class-I Hb mutants lowers the intracellular level of NO under hypoxia (Perazzolli et al. 2004) and pathogen attack (Seregelyes et al. 2004). Similarly, transgenic plants overexpressing class-1 Hb lead to the increased resistance to hypoxic stress and lowered necrotic symptoms in response to avirulent pathogens (e.g. tobacco necrosis virus and *Pseudomonas syringae* pv. *phaseolicola*). Therefore, the major activity of class-1 Hb is to provide defence system against lethal effects of nitrosative stress in order to assist and surpass the adverse conditions in plants. In addition to Hb and lipoxygenase, cytochrome oxidases (COX) are presumed targets of NO which is also mediated by metal nitrosylation and results in the loss of activity in biological system (Clark et al. 2000). Moreover, hundreds of studies have described the metabolic role of NO in plants, but no appreciation has been given earlier for the involvement of S-nitrosylation as a presumed post-translational protein modification. Currently advancement in methodologies has been successfully applied in mammalian cells (Greco et al. 2006), and this provides a better technique for isolating plant protein that are S-nitrosylated in vivo (Jaffrey et al. 2001). One

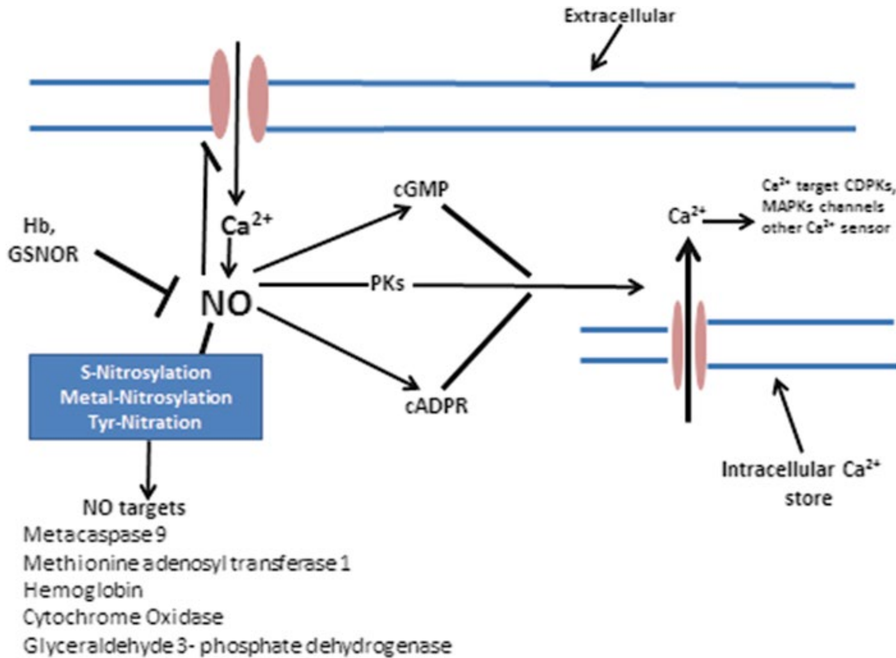


Fig. 1.11 Nitric oxide signaling in plant cells: NO convey its effects through S- Nitrosylation, metal- Nitrosylation and Tyrosine nitration. In these processes there is the involvement of Ca^{2+} channels. Protein kinases, cyclic ADP ribose and GMP mechanisms for NO signaling and switch off mechanisms include NO scavenging by Hb, GSNO (Nitrosoglutathione), reductase (GSNOR) and attenuation of Ca^{2+} influx that stimulates NO synthesis (Angelique Besson-Bard et al. 2008)

of the studies suggested that by the use of biotin switch method, the first S-nitrosylated proteins are isolated from the leaves of *Arabidopsis* and cell suspension extracts in which artificially released NO is applied (Lindermayr et al. 2005) and are found to be involved in metabolism, photosynthesis, redox control and stress response and various amongst these are known to be S-nitrosylated in vitro/in vivo in mammals (Stamler et al. 2001). By using biotin switch approach, the identification of methionine adenosyl transferase (MAT) is possible (Lindermayr et al. 2005). Many studies have revealed that the metabolic description for the NO to downregulate ethylene accumulation and some effects such as senescence forms the basal line of crosstalk between NO and ethylene signalling (Leshem et al. 2000). The interaction between NO and the *Arabidopsis* metacaspase 9 (ATMC9) provides the new insight for the effect of S-nitrosylation on plant protein activity (Belenghi et al. 2007). There are also evidences that show the presence of nitrosoglutathione reductase (GSNOR), conserved between bacteria, animals and plants (Sakamoto et al. 2002), and GSNOR catalyses the oxidation of GSNO to glutathione disulphide (GSSG) and ammonia (Liu et al. 2001) and hence plays an essential role by acting as checkpoints to regulate S-nitrosothiol-mediated effects in plants that are confronted by the pathogen (Rusterucci et al. 2007). The above finding reveals that the S-nitrosylation is an important post-translational reversible mechanism in plants.

Besides the above-defined signalling mechanisms, there are evidences that in animals Tyr nitration is basically involved with the knockdown of protein functions and is a relevant biomarker of NO-dependent oxidative stress (Hanafy et al. 2001), but recent studies have revealed that this plays a key role in post-translation modification in signalling (Schopfer et al. 2003). It has been observed that the protein kinase leads to the inhibition of Tyr phosphorylation, as Tyr nitration acts as antagonist to Tyr phosphorylation during

protein kinase-mediated cell signalling (Schopfer et al. 2003). Although in plants, association of ONOO– with Tyr residues in target proteinases has drawn the less information to date as mechanism of NO signalling. However the identification and isolation of highly reactive $ONOO^-$ in biological system have also been described, and various studies have revealed the active involvement of protein in Tyr nitration in plants. One of the recent studies has observed that by using the antibodies that are raised against 3-NO_2-Tyr residues demonstrates that there is 100-fold higher NR-mediated NO emission rate compared with the wild type that results due to the increased protein Tyr nitration in an antisense nitrite reductase tobacco lines. Similar results that have been observed from immunological-based strategy detected Tyr nitration in olive leaves exposed to salt stress (Valderrama et al. 2007) and also in tobacco cells treated with INFA, an elicitor secreted by Phytophthora infestans that promotes defence responses (Saito et al. 2006). All these proteins remain unidentified so far.

1.5.3 Role of NO and ROS in Plant Immunity

There are several evidences that described the role of NO and ROS in plant signal transduction; they act either individually or synergistically and stimulate a stronger defence response against various environmental stresses, and similarly the extensive generation of NO and ROS called as NO burst and ROS burst, respectively, has been identified to be involved in many morphological and metabolic processes such as tolerance against biotic and abiotic stresses and hormonal signalling and also plays an essential role in the plant growth and development (Hong et al. 2008). NO as a reactive radical is found to be involved in various essential processes in both plants and animals, and notably NO in animals is generated by NO synthase (NOS). Currently, NO generation as a radical and its diverse function in plant innate immunity has excited the plant biologist, as NO stimulates mitogen-activated protein kinase cascade (Kumar and Klessig 2000) and results in the increase of defence gene expression and other proteins markedly the enzymes coding for phenylalanine ammonia-lyase and pathogenesis-related proteins (Durner et al. 1998). Similarly the generation of NO in plants involves the reduction of nitrite by nitrate reductase, while oxidation mechanism by NOS of arginine to citrulline is not well defined. However, there are evidences that reveal that plants involve arginine-dependent pathway of NO synthesis, but there is no gene or protein in plants that resembles the mammalian-like NOS (Butt et al. 2003). From *Arabidopsis* a study revealed that the isolation of NOS-like enzyme (AtNOS1) has a sequence resembling a protein which is involved in the accumulation of NO in the snail Helix pomatia (Guo et al. 2003). But after some time it has been revealed that AtNOS1 protein does not exhibit NOS-like activity (Zemojtel et al. 2006), and therefore AtNOS1 was termed as AtNOA1 for NO Associated1 (Crawford et al. 2006), and a recent study reveals that AtNOA1 has circularly transposed GTPase activity in plastides (Moreau et al. 2008). Although *Arabidopsis thaliana* mutant noa1 is found to be useful for various physiological processes, it shows that only low levels of NO mediate plant growth, fertility, hormonal signalling, salt resistance and defence mechanisms (Kato et al. 2008). However, enormous generation of ROS results in the association between AtNOA and NO that is actually the outcome of the multiple effects and malfunctioning carried out by plastids (Gas et al. 2009). Decreasing or loss of function of NOA1 provides one of the simplest techniques for the analysis of NO function. The respiratory burst oxidase homology gene (RBOH) is a plant homology of NOX5 and has been found that in mammalian, NADPH oxidase is found in many plant genomes such as *Arabidopsis thaliana,* rice (*Oryza sativa*), tomato (*Solanum lycopersicum*), potato (*Solanum tuberosum*), tobacco (*Nicotiana tabacum*) and *Nicotiana benthamiana* (Yoshie et al. 2005). As it has been found that RBOH is present in several plants, at the same time it has also been observed that RBOH plays a crucial role in mediating ROS signal transduction that includes development, elongation and defence mechanism of the cell.

It has also been revealed that the genes AtRBOHD and AtRBOHF exhibit several properties that function in the generation of ROS during pathogen stresses (Torres et al. 2002) and ABA stimulating stomatal closing in guard cells (Kwak et al. 2003). It has been found that in *N. benthamiana*, the induction of NbRBOH A and NbRBOH B by virus-induced gene silencing (VIGS) results in the reduction of ROS generation that ultimately leads to the tolerance against *Phytophthora infestans* (Asai et al. 2008). Recent study showed that the use of antisense technology in the tomato plants results in the loss of RBOH function that reduces generation of ROS in leaves and leads to the phenotypic abnormalities (Sagi et al. 2004). It has been found that in root hair development AtRBOHC/RHD2 produced ROS which results in the stimulation of Ca^{2+} channels which in turn regulates cell elongation (Foreman et al. 2003). When act synergistically NO and ROS results in the cell apoptosis (Delledonne et al. 1998) while as normal generation of NO and H_2O_2 plays an essential role in hypersensitive response (HR) cell death (Delledonne et al. 2001).

1.5.4 Role of CDPK and MAPK as a Crosstalk in ROS

During pathogen signalling, the first step that arises in the cascade of signalling is the sharp increase of inward current of Ca^{2+} into the cytoplasm that results in the activation of ROS burst and HR cell death (Lecourieux et al. 2006). Recent studies have described the role of CDPKs in ROS and NO signalling mediated by various biotic and abiotic stresses and found that some CDPK genes are activated by certain transcription factors during pathogen signalling (Chico et al. 2002). It has been suggested that CDPK functions upstream in ROS generation (Kobayashi et al. 2007). It has been found that in tomato protoplast, the ectopic expression of AK1 (AtCPK1) in *Arabidopsis thaliana* accelerates NADPH oxidase activity and generation of ROS (Xing et al. 2001). During low abiotic-osmotic and wound signalling, there is an increased expression of NtCDPK2VK that activates generation of ROS and HR-like cell death and also found that under these stresses NtCDPK2VK accelerates the jasmonic acid, 12-oxo-phytodienoic acid and ethylene which has a crosstalk in ethylene signalling with MAPK (Ludwig et al. 2005). During the events like pathogen infection or elicitor treatment, there is quick activation of ROS phase I burst and then extensive ROS phase II burst and also described that a protein synthesis inhibitor is present in potato tubers and leaves that ends in ROS phase II burst (Yoshioka et al. 2001). In earlier studies a gene StRBOHA-D is isolated from potato plants (Yamamizo et al. 2006), and with the help of genetic engineering approaches in potato tubers, one gene StRBOHA is integrally expressed at low levels, and the other gene StRBOHB is activated from P. infestans by treatment with cell wall elicitor (Yoshioka et al. 2001). And also in leaves, the three genes StRBOHA, StRBOHB and StRBOHD are expressed at low levels, while StRBOHC is especially mediated in response to *P. infestans* (Yamamizo et al. 2006). Recent study of promoter analysis of StRBOHC reported that at the transcriptional level MEK2 plays an important role in the stimulation of gene expression and both I and II burst is abolished by NADPH oxidase inhibitor diphenylene iodonium, but another protein inhibitor cycloheximide ends only with phase II burst during the primary treatment of potato tubers (Kobayashi et al. 2007). These evidences suggest that StRBOHA and StRBOHB and StRBOHC contribute to phase I and phase II burst, respectively, and also inhibited by a protein kinase or a calcium inhibitor (Kobayashi et al. 2007). By in-gel kinase assays, it has been observed that StRBOHB acts as an important phosphorylation site and isolated Ser 82 and Ser 97 in the N-terminus of potato by using mutated *N*-terminal proteins of StRBOHB (Kobayashi et al. 2007). Also it has been observed that Ser 82 is phosphorylated in response to pathogen signalling in plants that is shown by using an anti-phosphopeptide (pSer 82) antibody. By cDNA expression screening, StCDPK5 is cloned by using the anti-pSer 82 antibody and found that the cells expressed an N-terminus of StRBOHB, and only Ser 82 and Ser 97 are phosphorylated by

CDPK in the *N*-terminus of calcium dependent which is analysed by mass spectrometry. ROS generation in leaves of *N. benthamiana* is stimulated by ectopic expression of StCDPK5VK, the integrally active mutant of StCDPK5. The ROS generation is stimulated by CDPK and is hindered by the loss of function of NbRBOHB in tobacco. The knockdown is complemented not by the mutant S82A/S97A but by the heterologous expression of wild-type potato StRBOHB. Phosphorylation of Ser 82 in tobacco is activated by the heterologous expression of StCDPK5VK. One of the studies suggests that the phosphorylation of RBOHs is mediated by StCDPK5 which in turn stimulates the ROS burst. These evidences have been analysed by bimolecular fluorescence complementation method which shows that intercommunication of StRBOHB and StCDPK5 has been carried out on the plasma membrane and revealed that the mutation of *N*-myristoylation and palmitoylation sites of StCDPK5 plays a crucial role in the membrane mobilisation, eludes these intercommunication.

Several studies have described that during plant-pathogen intercommunication, the generation of NO and ROS has several impacts on defence mechanism in plants and also exhibits various functions in defence systems that are mediated by these stresses (Fig. 1.12). It has been reported that *Arabidopsis thaliana* lacks the mutant AtNOA1 that results in the reduction of NO generation and, thus, increases the sensitivity of noxious Pseudomonas syringae (Zeidler et al. 2004) indicates that NO is participating in basic defence system during compatible hemibiotrophic bacterial pathogen and Arabidopsis interaction. A study showed that silencing of NbRBOHB in *N. benthamiana* results in the adverse impact on tolerant property against potentiate pathogen *P. infestans* (Asai et al. 2008) but not to *B. cinerea*, and by silencing NbNOA1 it leads in the stimulation of extreme high sensitivity to *B. cinerea* but not to *P. infestans* (Asai and yoshioka 2009). It has been observed that expressing StCDPK5 fused to pathogen-inducible promoter in potato plants shows high tolerance to *P. infestans* but shows high sensitivity to necrotrophic pathogen *Alternaria solani* (Yoshioka et al. 2009). With the help of genetic engineering approaches, it has been found that StCDPK5 is able to generate ROS but incapable of producing NO which implies that ROS may have a negative role in disease resistance to necrotrophic pathogen or a positive role in expansion of disease lesions. It has been observed that expressing StMEK2 (StMEK2^{DD}) and a pathogen-inducible promoter gene StCDPK5VK in the transgenic potato plants show resistance to both *P. infestans* and *A. solani* (Yamamizo et al. 2006). One of the recent studies showed that SIPK is activated by MEK2^{DD} which results in the generation of RBOH-dependent ROS and NO production which is stimulated by NOA1 in *N. benthamiana* (Asai and Yoshioka 2008). As it was found that MAP kinase plays an important role in NO and ROS signalling, they also play a crucial role in the activation and phosphorylation of nitrate reductase (Wang et al. 2010). The above findings revealed that contribution of NO in plants makes them resistant against necrotrophic pathogens *A. solani* and *B. cinerea* besides other diseases, but simultaneously NO together with ROS induces apoptosis which is an advantage over these necrotrophic pathogens. This implies that NO and ROS synergistically mediates a stronger response against these environmental stresses. It has been observed from the earlier studies that NO has several functions along with ROS that contributes to either cell apoptosis or resistance to a number of pathogens depending upon the type of stress that leads to the open question of how NO and ROS changes the physical and cellular metabolism. It is not always possible that generation of ROS leads to increased sensitivity of the cell which depends on the exposure and intensity of ROS formation, but it can also result in the activation of defence responses during various abiotic and biotic stresses.

1.6 Perspectives and Conclusion

Plants have developed various stress-tolerant mechanism during their evolutionary period of development. In recent times plant signal transduction has been in focus and has emerged as an

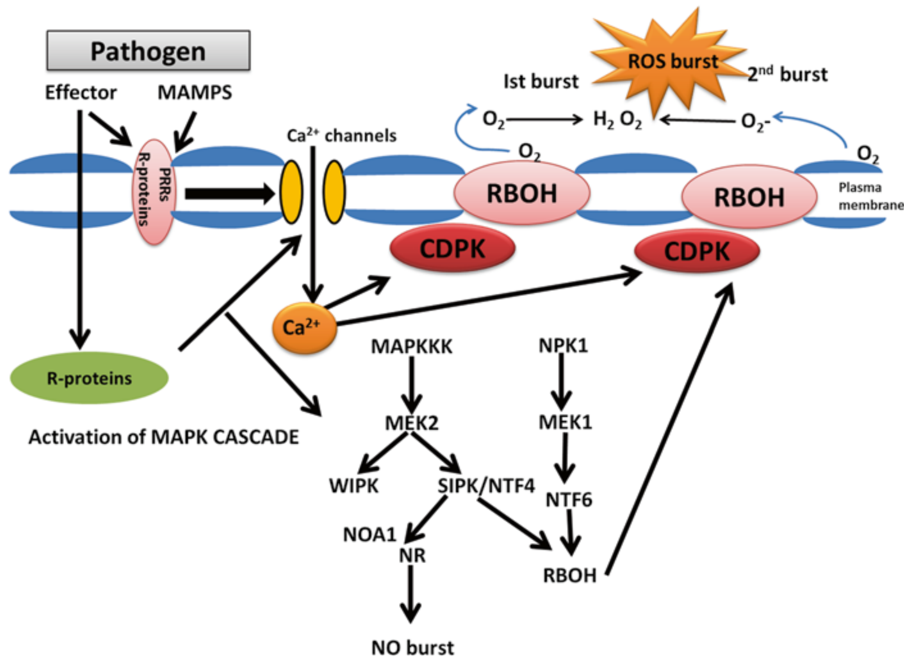


Fig. 1.12 A model showing NO burst and ROS burst that induces Ca^{2+} channels, CDPKs and MAPK cascade that upregulates inducible form of RBOH gene (Hirofumi Yoshioka et al. 2011)

important area of research in biology. The plants mediate responses to these environmental changes through diverse mechanisms that help them to withstand these changes. The role of calcium-mediated signalling pathways in plants has been investigated and shows gross resemblance to that of animal system. As CDPKs play an essential role in signalling pathways, therefore, there is a need for broad and comprehensive knowledge regarding their structural and functional relationships. Through various studies it has been revealed that there is an active involvement of MAP kinase in plant signal transduction. In majority of cases, only indirect proof regarding the involvement of MAP kinase in signalling is known but the responsible genes/ protein kinase that regulates these signalling pathways is still unknown. In addition to this, other types of protein kinases also share some properties of MAP kinases such as substrate, specificity and size. Therefore, utilising the proficient technologies to identify the particular MAP kinases and their respective genes encoding enzymes is of fundamental importance to biology. Therefore, multidisciplinary approaches are required to develop novel methods of analysis just to acquire better understanding about the function of MAP kinase in different processes. Further, identification and characterisation of plant protein kinases and their intercommunication will lead to insights into the mechanism regulating plant growth and development. ROS formation during various metabolic reactions poses a deleterious effect on different cellular components by causing disruption of the electron transport chain. ROS play two different roles in plants, viz. at low concentration they behave like signalling molecules mediating several plant processes, while at high concentration they cause oxidative stress leading to apoptosis. In response to oxidative stress, plants have developed endogenous defence mechanisms comprising of enzymatic and nonenzymatic antioxidants. Despite that much progress has been made in current years, there are still some lacuna in ROS formation and its ill effects on plant growth and development because of their short half-life and high reactivity. With the help of advanced

analytical approaches, the fate of ROS formation will assist in developing a broad knowledge about their function in cellular signalling. Last but not least, future research should be focused more on biochemical, molecular and genomic technologies to better understand the signalling pathways in plants that will ultimately lead to the significant advancement in the fields of agricultural, biotechnology and forestry.

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2 SNARE Proteins as Signaling Elements

Reiaz Ul Rehman and Gian-Pietro Di Sansebastiano

Abstract

SNAREs (*N-ethylmaleimide-sensitive* factor adaptor protein receptors) are small polypeptides (~200–400 amino acid) which are characterized by a particular domain, the SNARE motif that can form a coiled-coil structure via hetero-oligomeric interactions. These protein interactions are highly stable leading to the formation of the so-called SNARE complex which allows the membrane fusion. SNAREs also interact with several proteins acting as regulators of SNARE complex formation. By regulating vesicle traffic, SNAREs have a clear influence on several signaling pathways. SNAREs take part to receptors turnover through endocytosis and exocytosis, but they can also directly gate channels and interact with membrane proteins potentially involved in signaling processes. Phosphorylation of SNAREs upon elicitation is known, and hormonal control confirms that SNAREs have a role in signaling processes.

Keywords

SNAREs (*N-ethylmaleimide-sensitive* factor adaptor protein receptors) • Endocytosis • Exocytosis • Endomembrane system • Synaptobrevins

2.1 Introduction

Eukaryotes have evolved a sophisticated system within the endomembrane system for extracellular transport of cargo molecules from the endoplasmic reticulum (ER) via the Golgi apparatus. The small membrane-coated transport vesicles shuttle between the compartments (including ER, Golgi apparatus, plasma membrane, and vacuoles) of the secretory pathway. The plant vacuoles are complex and multifunctional organelles that serve diverse functions such as storage, digestion,

R. Ul Rehman
Department of Bioresources, University of Kashmir, Srinagar 190006, Jammu and Kashmir, India
e-mail: reiazrehman@yahoo.co.in

G.-P. Di Sansebastiano (✉)
Di.S.Te.B.A., Università del Salento, via prov. Lecce-Monteroni, 73100 Lecce, Italy
e-mail: gp.disansebastiano@unisalento.it

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and recycling, and unlike in yeast the functional vacuoles present are essential for plant cells (Rojo et al. 2001). The vesicles in addition to extracellular transport, i.e., anterograde transport (exocytosis), are also required for uptake of material from the extracellular space, i.e., endocytosis and other retrograde transport processes. There are several classes of polypeptides that contribute in these shuttling activities, and the fusion process between a coated vesicle and a target membrane is an unfavorable process in terms of energy. The eukaryotic systems have evolved a specialized class of proteins that drives the membrane fusion and these are named as soluble *N*-ethylmaleimide-sensitive factor adaptor protein receptors (SNAREs). The SNAREs provide the force to bridge membrane lipid bilayers together and provide the specific matching specificity between vesicles and targeted compartments. In doing so they contribute to targeting and delivery of membranes and soluble proteins in all eukaryotic cells (Lipka et al. 2007).

The role of SNAREs in relation to membrane trafficking is not only limited to general homeostatic and housekeeping functions, but it also represents important signaling and response elements associated with growth, osmotic stress, gravitropism, and defense. The first evidences arrived with the discovery of the tobacco SNARE NtSYP121 (=NtSYR1) and its homologue in *Arabidopsis*, and this protein was found involved in abiotic stress signaling (Leyman et al. 1999) and, later, in pathogen resistance (Collins et al. 2003).

The canonical model of SNAREs function describes them as complementary sets of interacting proteins which target vesicles to the specific destination membrane. This model has to be enriched by the evidences of their contribution in scaffolding and anchoring of other membrane proteins that play their roles in response to environment, in growth as well as development. In comparison to animals and fungi, the plants have a larger number of SNAREs. In contrast to the members of the former kingdoms, the plants are lacking some of the particular SNARE protein subfamilies but have additionally evolved some novel types of SNAREs. After the description of the canonical model, this chapter will review the latest evidences about the relation of SNAREs with signaling in the plant cell.

2.2 SNARE Structure and Function

2.2.1 The Canonical Model

The SNAREs comprise of a large superfamily of relatively small polypeptides (~200–400 amino acids) which are characterized by the presence of a particular domain, the SNARE motif (Jahn and Scheller 2006). This domain has a stretch of 60–70 amino acids which consists the heptad repeat forming a coiled-coil structure. The SNAREs form highly stable protein-protein interactions via the hetero-oligomeric interactions that facilitate in overcoming the energy barrier required for membrane fusion. The SNAREs are required for mediating the fusion events between membranes in vesicle-associated traffic, and for performing this function they are thus distributed on vesicles and the organelles of the endomembrane system, including the plasma membrane.

The SNAREs possess *C*-terminal transmembrane (TM) domains that usually help in its association with membrane bilayer (Fig. 2.1a). The SNAREs that lack the TM domains are associated or attached with the membranes via the lipid anchors. There is an exception to possession of one SNARE motif, viz., SNAP-25-like SNARE protein that contains two SNARE domains that are separated by a flexible linker (Fig. 2.1a). In addition to the SNARE domain and the *C*-terminal TM domain, many SNAREs have an *N*-terminal region with regulatory function that generally controls the activity of SNARE protein and coordinates its activity with several accessory polypeptides (Fig. 2.1a).

The membrane fusion is mediated by interactions among the complimentary SNAREs associated with the vesicles and the target membrane. Once the SNAREs are matched, they form a highly stable association known as the “SNARE complex.” This complex comprises of three

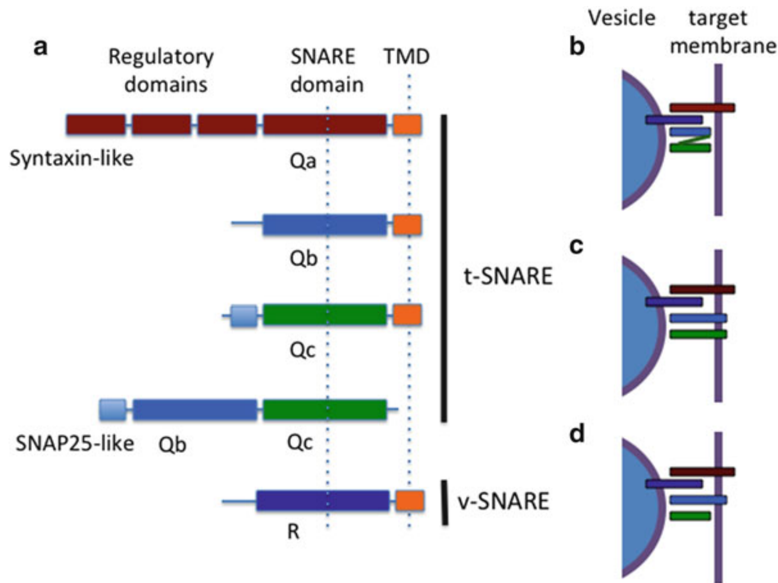

Fig. 2.1 The figure proposes a simple representation of SNARE structure and assembly. (**a**) Organization of Q- and R-SNARE domains; (**b**) SNARE complex composed by three proteins, typically observed in synaptic transport (Syn1/Vamp/SNAP25) and other transport event on the PM; (**c**) SNARE complex composed by four proteins, typically observed on yeast ER and endosomes (Syn7/Vamp/Syn8/VTi1); (**d**) SNARE complex composed by four proteins, typically observed on yeast vacuoles (Vam3/Vamp/Vam7/VTi1)

or four types of distinctive SNARE proteins which contribute to form a four-helix bundle of intertwined SNARE domains (Brunger 2005; Jahn and Scheller 2006).

The classification of SNAREs is based on either their localization (functional classification) or on the basis of the presence of specific amino acids in the center of the SNARE motif (structural classification). On the basis of functional classification, SNAREs are divided into vesicle-associated (v-SNAREs) and target membrane-associated SNAREs (t-SNAREs) (Lipka et al. 2007). This classification does not take into account the role of SNAREs in the context of homotypic fusion events or progressive anterograde traffic. The structural classification of SNAREs has been indicated as Q- and R-SNAREs based on the presence of either a conserved glutamine or an arginine residue in the middle of the SNARE domain (Fasshauer et al. 1998). Further, the functionally classified t-SNAREs correspond to the structurally classified Q-SNAREs, and similarly functionally classified v-SNAREs correspond to the structurally classified R-SNAREs. The target membrane-localized Q-SNAREs are of three types which are further subdivided into Qa-, Qb-, and Qc-SNAREs. The SNAP-25-like proteins of Q-SNAREs constitute a special class with both Qb- and Qc-SNARE motif. Historically the SNAREs are often designated and described on the basis of their role in synaptic exocytosis, and thus the Qa-SNAREs are frequently called as syntaxins (Bennett et al. 1992) and vesicle-resident R-SNAREs are called as VAMPs (vesicle-associated membrane proteins). The R-SNAREs can either have a short or a long *N*-terminal regulatory region, gaining the designation of brevins (lat. brevis, short) and longins (lat. longus, long). The brevins for their role in synaptic exocytosis have been frequently called as synaptobrevins; however, this evolutionary class of R-SNAREs is absent in plants, and thus all R-SNAREs present in the plants are only longins (Uemura et al. 2005).

2.2.2 SNARE Genomics in Plants

Several SNAREs have been located in the genomes of all higher plant species as such that there are 60 SNAREs in dicotyledonous model species *Arabidopsis thaliana* (The Arabidopsis Genome Initiative 2000), 57 SNAREs in monocotyledonous *Oryza sativa* (International Rice Genome Sequencing Project 2005), and 69 SNAREs in the *Populus trichocarpa* (Tree black cottonwood: Tuskan et al. 2006). In contrast the yeast *Saccharomyces cerevisiae* encodes for 21–25 SNAREs, and the humans (*Homo sapiens*) are thought to encode 35–36 SNAREs (Jahn and Scheller 2006; Sutter et al. 2006).

There are comparable numbers of SNAREs in the same subfamily in various plant genomes that suggest that the enlarged SNARE number, compared to other eukaryotes, is not related to a particular plant lifestyle or habitat but related to an essential aspect of plant biology. The higher number of SNAREs found in plant species as compared to fungi and animals is predominantly thought to be due to the expansion of number of members in conserved SNARE subfamilies and not due to the evolution of new isoforms. There are only two subfamilies which appear to be plant specific: the novel plant-specific SNARE (NPSN) Qb- and the SYP7 Qc-SNAREs (Sanderfoot et al. 2000; Lipka et al. 2007). The presence of most of these SNARE genes in green algae *Chlamydomonas reinhardtii* and moss *Physcomitrella patens* also indicates that these essential characteristics evolved early in plants to satisfy the necessity which arose for plant-specific biological processes. There are many plant-specific processes such as a specific type of cytokinesis, gravitropic responses, and phytohormones transport, and several of these processes are related to signaling. As well as in other eukaryotes, many of these functions are also involved in establishing and maintaining cellular processes polarization (Surpin and Raikhel 2004).

2.2.3 Q-SNAREs

Plant genomes encode multiple syntaxin-like isoforms called SYP (syntaxin of plants) subfamilies. These SNAREs were originally intended and indicated with an *N*-terminal autoregulatory domain, a linker, the SNARE domain, and a TM region (Fig. 2.1). This description has to be considered as a general indication with several exceptions in terms of autoregulatory and transmembrane domain. The examples being SYP5, 6, and 7, which were initially grouped as syntaxins but are now being regarded as Qc-SNAREs even if they are still designated as syntaxins (Pratelli et al. 2004; Sutter et al. 2006). The SNAREs that better correspond to the original description of syntaxins are all the Qa-SNAREs, but they also possess a natural genetic polymorphism. For example, AtSYP23 a Qa-SNARE lacks the *C*-terminal TM domain in the *Arabidopsis* ecotype Col-0 (Ohtomo et al. 2005). The *N*-terminal auto-inhibitory domain in AtSYP23 is composed of three helices which are also called the Habc motif, and this domain is folded into three helical bundles which mimics the parallel four-helix bundle of the SNARE complex. Furthermore, the folded domain interacting with the Q-domain in the so-called close conformation prevents undesired interaction of the protein with the partners before activation (Munson et al. 2000). The plant Qa- and Qb-SNAREs have been reported to have roles in several biological processes such as shoot gravitropism, cytokinesis, and autophagy. However up until now, no phenotype was revealed upon genetic screens for a Qc-SNARE. The Qb- and Qc-SNAREs are also known to possess an extended *N*-terminal domain occasionally, and this domain may also adopt (in animals) a coiled-coil structure similar to a Habc motif (Hong 2005). For example, the SNAP-25 comprises of 2 SNARE motifs and thus is classified as a Qb+Qc-SNARE. The Qb-domain of SNAP-25 is equivalent to *N*-terminal domain, and the Qc-domain is equivalent to a *C*-terminal domain. Moreover, the *Arabidopsis* SNAP-25-like proteins also lack a TM domain (Lipka et al. 2007). The mammalian SNAP-25 is attached to the PM by palmitoylation (Veit et al. 1996); however, the *Arabidopsis* SNAP-25s do not have an appropriate conserved palmitoylation sites (Lipka et al. 2007). Nevertheless, the AtSNAP33 has been reported to localize to the PM (Wick et al. 2003) more like the animal homologue (Hong 2005).

2.2.4 R-SNAREs

The R-SNAREs are grouped in three subfamilies, the SEC22s, VAMPs, and YKT6s. These are located mostly on trafficking vesicles and are anchored to them by the *C*-terminal TM domain. In animals and yeast the R-SNAREs, AtYKT61, and AtYKT62 are attached to the vesicular membranes by lipid anchors which are added to these posttranslationally (McNew et al. 1997). The plant R-SNAREs can be classified as longins because of the presence of longin domain (an extended *N*-terminal stretch). This longin domain is responsible for subcellular localization as well as regulation of SNARE complex assembly in other eukaryotes (Lipka et al. 2007). When compared to Q-SNAREs the information about the biological roles of plant R-SNAREs is scarce. However, there is only one exception of a recently discovered salt resistance phenotype (Leshem et al. 2006).

2.2.5 Expression and Subcellular Localization

The availability of microarray data (https://www.genevestigator.ethz.ch) allows the expression profiling of SNARE isoforms in various plant cell types, tissue, and organs. The expression of many SNAREs is ubiquitous, in particular SYP22 and SYP32 (Qa-SNAREs); VTI11 and GOS12 (Qb-SNAREs); BET11, SYP71, SFT11, and USE11 (Qc-SNAREs); SNAP33 (Qb+Qc-SNARE); and VAMP713, VAMP714, VAMP721, and VAMP722 (R-SNAREs) (Lipka et al. 2007).

Interestingly many SNAREs have apparently a relatively maximum transcript accumulation in pollen. This fact supports the idea that for the pollen development and function, a comprehensive vesicle-associated transport processes are required which are essential to pollen tube: cell polarity and tip growth (Hepler et al. 2001). Mostly the SNAREs are associated with specific membranous compartments, but some of them are localized to two or more distinct organelles which are possibly due to their shuttling between various subcellular compartments. The overexpression studies utilizing the full-size SNAREs or the cytoplasmic SNARE domains have provided fresh insights into the potential role of these isoforms in vesicle trafficking.

2.2.6 Regulators and Associated Proteins

The SNARE proteins are able to drive vesicle fusions in vitro; however, in vivo these SNAREs are not the only determinants of vesicle fusion and targeting specificity. For the formation of SNARE complex, the SNAREs are known to interact with many proteins which act as the regulators (Lobingier and Merz 2012; Schafer et al. 2012). The regulatory factors Sec1/Munc18 (SM) proteins are also important determinants interacting with non-conserved SNARE domains which control the conformational changes of syntaxins *N*-terminal Habc domains. The genome of *Arabidopsis* is reported to contain six members of the Sec1 family, and out of these one of the member which is called as KEULE (KEU) was shown to be involved in cytokinesis (Assaad et al. 2001). Another important class of regulatory proteins is the Ras-related GTPases belonging to the Rab GTPase family, and these are involved with SNAREs in controlling the multiple steps of vesicle transport. The *Arabidopsis* has 57 Rab GTPases classified in eight subfamilies (RabA to RabH) (Rutherford and Moore 2002). The regulatory role of Rabs interactions with SNAREs or the multi subunit protein complex called exocyst complex will be treated in a separate chapter. For the specificity of membrane fusion, the SNAREs may also fine-tune their function by partly acting as inhibitory SNAREs (i-SNAREs) (Sansebastiano 2013). In this action it may either substitute or bind to the fusogenic SNARE protein and thus forming a non-fusogenic complex (Varlamov et al. 2004). These later possibilities remind the importance of in vivo SNAREs stoichiometry in membrane fusion events (Fig. 2.2).

Further, for the process of SNARE recycling, the soluble accessory proteins, viz., *N*-ethylmaleimide-sensitive fusion protein NSF (an ATPase) and alpha-SNAP, are required. The interaction of an NSF with the SNARE complex

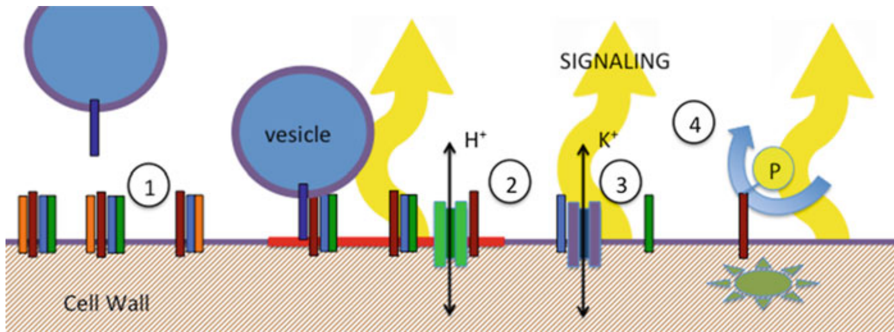

Fig. 2.2 SNAREs are involved in signaling in different ways. SNARE stoichiometry tells us they are more abundant than required for membrane traffic; they assemble also to form non-fusogenic complexes (1) and to interact with proton pumps to define membrane microdomains and potential (2) and other unknown partners; SNARE influence turnover of channels, but it is known that gating of Ca^{2+} and K^{+} depends on SNAREs through direct protein-protein interactions (3); PM SNAREs can be phosphorylated as part of the signaling cascade elicited by interaction with microorganisms or hormonal stimulation (4)

takes place via the alpha-SNAP resulting in hydrolyzing ATP and dissociation of the complex (Barnard et al. 1997). *Arabidopsis* has three genes for alpha-SNAP and one for NSF, but nothing is known about their specific biological role (Sanderfoot et al. 2000).

2.3 SNAREs and Signaling

2.3.1 Development

The gravity direction is sensed by the plants which affect their growth orientation. The gravitropic responses are controlled by complex molecular mechanisms which involve signaling (perception and transduction) and growth adjustments. Initially during the gravity perception, the starch-filled amyloplasts undergo the changes in sedimentation, and these organelles are present in the endodermis (statocytes) of shoots and columella cells (statocytes) of root cap. They have an influence over the signal transduction cascades that are involved in auxin transporters relocalization resulting in altering of auxin flux. This alteration causes the compensatory asymmetric growth responses that are largely reliant on the vacuoles (Lipka et al. 2007; Bassham and Blatt 2008). Many gravitropic responsive genes have been isolated, and in *Arabidopsis* plant the Qa-SNARE AtVAM3/SYP22 (*SGR3*) and the Qb-SNARE AtVTI11 (*ZIG*/*SGR4*) of the SNARE complex play an important role in shoot gravitropism (Yano et al. 2003). Further the v-SNAREs comprising of VTI1 group are the best studied among the SNARE subfamily. The single mutations in VTI1 were shown to be viable while as the double mutations were embryo lethal (Surpin et al. 2003). The mutants with single mutation were having minor defects which placed them into two distinct phenotypes, viz., *vti11* and *vti12*. The knockout mutant's *vti11* and *vti12* showed developmental and growth-related defects. The *vti11* showed defects such as vascular patterning, auxin transport, and shoot gravitropism (Kato et al. 2002; Surpin et al. 2003) while as the *vti12* mutants were hypersensitive to starvation, and these showed premature senescence (Surpin et al. 2003) similarly to autophagy mutants. The studies on VTI11 and VTI12 revealed that the vacuolar trafficking is affected as such that in *vti11* mutant showed defects in protein trafficking to lytic vacuoles (LV), while as the *vti12* mutant showed defects in storage protein transport to protein storage vacuoles (PSV). The specificity of function in the single mutants, viz., *vti11* and *vti12* means that there can be partial substitution of proteins in SNARE complexes or in other words the redundancy of function (Sanmartín et al. 2007). The mechanism that allows SNAREs

to respond to gravity is unknown; however, there is a possibility that the mutant phenotype arises because of an indirect effect on the structure and composition of the vacuolar membranes rather than the direct effect of vesicle trafficking on gravitropism. There is an abnormal vacuolar structure in these mutants with an absence of transvacuolar strands and accumulated presence of vesicle-like structures which may be possibly impeding the amyloplast movement and may also be interacting with the cytoskeleton.

Membrane traffic affects growth most probably by altering the correct sorting of membranes and cell wall components. Tip growth in pollen tubes can be taken as a good example of growth processes because there exists a continuous vesicle secretion as well as the delivery of new wall material. It was recently shown that SNAREs have probably a determinant role in the pollen growth processes. The localization of pollen-specific syntaxin SYP125 (*AtSYP125*) was shown to be associated with the plasma membrane (PM) and apical vesicles in growing pollen tubes. The AtSYP125 was asymmetrically localized behind the apex at the plasma membrane, while as AtSYP124, another pollen-specific syntaxin was distributed differently (Rehman et al. 2011; Silva et al. 2010). Thus, the syntaxins asymmetric distribution in pollen tubes helps to define the exocytic sub-domains; however, there is also the requirement and role of other signaling and functional mechanisms such as phosphoinositides and small GTPases. Membrane traffic also regulates the transport capacity of selected ion and solute transporters (2, 3 in Fig. 2.2). Among these the most characterized in mammalian cells is the trafficking of GLUT4 (Na^+-coupled Glc transporter). It has been shown in the intestinal epithelial cells that GLUT4 cycles between apical membrane and cytosolic vesicle pool. The SNARE complexes involved in fusion of GLUT4 vesicles include mammalian syntaxin 4, SNAP-23, and VAMP2 within the lipid rafts of the plasma membrane. The recovery of these GLUT4 transporters form the apical plasma membrane takes place by endocytosis, and the sequestering takes place in specialized GLUT4 vesicles before recycling occurs (Grefen and Blatt 2008). This example shows how the traffic is characterized by changes in the integral membrane proteins turnover. Unlike GLUT4, many other proteins follow the path leading to the vacuole for final degradation after endocytosis. There is no information about the molecular mechanics of this trafficking; however, SNAREs do certainly play a role. Another interesting example is the traffic of the KAT1 (Kv-like K^+ channel) of the epidermal cells whose turnover at the plasma membrane is tightly controlled through a mechanism evoked by ABA that leads to recycling in an endomembrane pool that is distinctive from the degradation pathways leading to the vacuole. The studies using the dominant negative fragments of syntaxin SYP121 have revealed that the KAT1 transport to the plasma membrane depends on SYP121 function (Grefen and Blatt 2008). The lateral mobility of KAT1 also increased greatly (100-fold) in the presence of dominant negative fragments, and this is an indicative of an additional role of SNARE helping to anchor the KAT1 protein present within the lipid microdomains (2 in Fig. 2.2). Recent work directly evidences that SYP121 is the key structural element which determines the gating of another K^+ channel (Grefen et al. 2010) which makes it clear that in fact SNARE is part of a scaffold of proteins that is associated with membrane transport of K^+. Thus, the SNARE may be essentially required for channel-mediated K^+ nutrition that is a wholly distinct function from other roles in membrane traffic. In fact a few SNARE proteins such as in mammalian nerve cells, the syntaxin 1A is known to interact with ion channels binding several Ca^{2+} and K^+ channels.

2.3.2 Hormones

In the plants the vesicle trafficking has been implicated in a variety of responses pertaining to hormonal and environmental stimuli. This paragraph can only make limited examples about the existing cases of study. For example, ABA signaling was shown to be related to evoked endocytosis at the plasma membrane which ably selected among the integral membrane proteins and also

regulated their recycling back to the plasma membrane. The phytohormone ABA is pivotal in regulating cellular responses to abiotic stresses, and it acts as a signal that rapidly triggers changes in three K^+ and Cl^- channels such as in the control of ion transport of stomatal guard cells resulting in transpiration suppression from the leaf tissues. Concurrently, ABA is known to initiate endocytosis of KAT1 K^+ ion channel which is otherwise normally active during the K^+ uptake for stomatal opening. Furthermore, a study on a cDNA screen in frog (*Xenopus laevis*) oocytes for ABA-related genes ultimately led to the identification of a *Nicotiana tabacum* syntaxin NtSYR1 (which is an ortholog of *Arabidopsis thaliana* AtSYP121/AtSYP122). The SYP121 or the close homologue SYP122 contributes in early changes during ion channel gating in ABA response. It has been shown that the SYP121 and SYP122 dominant negative fragments were blocking changes in K^+ and Cl^- channel gating in response to ABA (Leyman et al. 1999; Geelen et al. 2002). The changes due to action of the dominant negative fragments can lead to the rise in intercellular calcium $[Ca^{2+}]_i$ due to suppression of the Ca^{2+} channel gating and its entry across the plasma membrane. The fact that in the guard cells K^+ and Cl^- channel currents are controlled mainly by the $[Ca^{2+}]_i$, it underscores the importance of the mechanisms regulating the integration of SNARE with ion channel regulation during the process of stomatal closure. Further, it also raises similar questions about integration and role of Ca^{2+} signaling with other physiological responses such as the responses to pathogens (see following paragraph) (Grefen et al. 2010, 2011). In one such response studies, the genetic screening in *Arabidopsis* for altered salt tolerance identified a knockout line [*osm1* (osmotic stress-sensitive mutant 1)] in which the T-DNA insertion was found to be similar to SYP61, and it co-segregated closely with osm1 phenotype and was found to be only a functional mutant (Zhu et al. 2002). In the root bending assay, the mutant showed sensitivity towards osmotic stresses (both ionic and nonionic). It also showed the hypersensitivity towards the drought stress, and the stomata showed insensitivity towards the ABA-induced opening and closing. However, it was surprising to note that upon the expression of antisense *AtVAMP711*, there was an improved salt tolerance in the above mutant lines. Similarly, the individual T-DNA insertional lines of *AtVAMP711*, *AtVAMP713*, and *AtVAMP714* also exhibited the improved salt tolerance. This phenotype coincided with a failure of osmotic stress-induced, reactive oxygen species-containing endosomal vesicles to fuse with the central vacuole. Thus the SNAREs can then have an antagonistic function in abiotic stress responses.

About the contributions of SNAREs to trafficking associated with auxin, some intriguing ideas emerged from the study of the PIN (for PIN-formed) and AUX1 (for AUXIN1) proteins. The SNARE proteins in the mammalian epithelial cells are responsible for the differential targeting of solute transporters (e.g., Na^+/K^+-ATPase, gastric H^+-ATPase) and the coupled transporters (e.g., GLUT4:Glc transporter) to apical and basal cell membranes. Considering the analogy to mammalian epithelial polarity to be true, it was expected that the AUX1 (an auxin uptake carrier) and PIN (an auxin efflux carrier) traffic will be dependent on the different subsets of Q- and R-SNARES because the AUX1 is localized on the apical ends of the cells (in *Arabidopsis* epidermis and cortex of stem and root), while as the PIN1 protein is present on the opposite ends of the same cells. However, these results are still controversial and need further clarification (Grefen and Blatt 2008).

2.4 SNAREs in Plant Interaction with Microorganisms

The SNAREs have been reported to have several roles in plant defense responses against the pathogen attacks (Reichardt et al. 2011). The resistance of *Arabidopsis* to powdery mildew of barley (*Hordeum vulgare*) is the best characterized example. On the *Arabidopsis* (a nonhost plant) the spores of the powdery mildew are able to germinate but are not able to penetrate the cells and thus are unsuccessful in establishing an infection. This is an example of nonhost resistance which is

an active mechanism where the cell wall provides a physical barrier at the site of the pathogen attempting to penetrate by secreting deposits known as papillae (Kwon et al. 2008). The genetic screening of *Arabidopsis* identified a defective nonhost resistance mutants called as pen mutants (having increased penetration of pathogen), and among these pen1 mutant was discovered with mutation in SYP121 gene. To find again SYP121 may look surprising, but biotic and abiotic stress have many common points. However, the precise function of SYP121 in pathogen resistance is not yet clear. In barley it has been seen that the cells below the site of infection have hydrogen peroxide filled in their vesicles, but these vesicles were decreased in *ROR2* mutant (*SYP121*-homologue). Due to the decrease in vesicles in *ROR2* mutants, they were more susceptible to fungal penetration, because of the fact that vesicles potentially function in cross-linking of the cell wall components in response to the attempts of infection. It was also found that the *Arabidopsis SYP121* was more susceptible to infection due to the delay in fungus-induced formation of papillae. These mutants also had an increase in the expression of PR-1 (pathogen response gene) and SA (salicylic acid). The increase in SA (a signal molecule in defense pathway) in these mutants suggests that SYP121 acts as a regulator of SA-mediated defense pathway. These findings indicate that SYP121 may have distinct and opposite roles in modulating several different pathogen-responsive pathways.

Another SNARE SYP122 is regulated in a different manner in spite of being very similar to SYP121 (Grefen and Blatt 2008). The expression of SYP122 is induced by viral, bacterial, and fungal infections. In response to flg22 (a bacterial elicitor) it showed rapid phosphorylation that may suggest its role in pathogen defense like its compatriot SYP121. However, the SYP121 phosphorylation takes place in response to Avr9 (race-specific elicitor) and not flg22 (4 in Fig. 2.2). Furthermore, the SYP121 mutants did not show any detectable defects in disease resistance like the SYP121. The double-mutant SYP121/122 indicated overlapping of individual function of SYP121 and SYP122. Further, unlike either of the mutants, the double mutant SYP121/122 was dwarfed, developed necrosis in patches, and had higher levels of SA and PR-1. These studies suggest that SYP121 and SYP122 are functionally distinct (Rehman et al. 2008) and they can partially substitute for one another.

A third plasma membrane syntaxin that was the object of deeper studies, SYP132, has also been implicated in defense response against the bacterial infection. It is also phosphorylated upon elicitor treatment and thus suggesting that phosphorylation may be a general phenomenon required for regulation of defense-related SNAREs. Probably many of the secretory SNAREs are multifunctional proteins which under the normal conditions are having a role in the general secretory pathway. But during the pathogen attack, they are recruited to defend the cells and are required for delivery of cell wall material and defense proteins during the infection. In fact the expression of *SNAP33* a Qa+Qb partner of both SYP121 and SYP122 is also induced by pathogen attack, and its knockout mutants are dwarfed and develop necrotic lesions.

2.4.1 Plant-Symbiont Interactions

During the endosymbiotic interactions the host forms the specialized membrane compartments. There are two well-studied situations which are of agricultural as well as ecological relevance: firstly the arbuscular mycorrhizal symbiosis and the second rhizobium-legume symbiosis. In both of these cases the host produces the specialized membrane surrounding the microbes to form a symbiotic interface which facilitates the exchange of nutrients. Despite their importance, the mechanisms for the formation of these specialized membrane interfaces are largely unknown (Lipka et al. 2007). There is little information about the role of SNAREs in these symbiotic relationships. In the legume species (*Lotus japonicus*) it has been reported that one of the *LjSYP32* isogenes (*LjSYP32-1*) which encodes orthologs of the AtSYP32 syntaxin appears to function in root nodule development. The *AtSYP32* and

LjSYP32-1 are expressed ubiquitously but having a preference for roots. The transgenic antisense *Lotus* lines proved that this protein has in fact a role in plant development as well as root nodule organogenesis.

The *Medicago truncatula* MtSYP132, an ortholog of AtSYP132, was shown localized in root nodules in the specialized symbiosome membranes of the so-called infection threads and infection droplets. Finally, the R-SNARE exocytotic vesicle-associated membrane proteins (VAMPs) are also required for the formation of the membrane at the symbiotic interface in both interactions.

2.5 Conclusion

We have seen SNAREs taking part to receptor turnover through endocytosis and exocytosis, we have seen them gating channels (e.g., KAT1), and recent experiments also suggest the regulation of proton pumps activity. These mechanisms have a clear effect on signaling. Moreover SNAREs phosphorylation upon elicitation and hormonal control confirm that future studies on signaling will find more and more evidences about the role of these proteins in all signaling processes.

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3 Plant Rab GTPases in Membrane Trafficking and Signalling

Reiaz Ul Rehman and Gian-Pietro Di Sansebastiano

Abstract

In the eukaryotic systems the membrane trafficking inside the cells is indispensible. The membrane trafficking is a highly regulated process in which various molecular machineries are involved. It involves the vesicle formation, tethering, and finally fusion. According to the phylogenetic analysis, these processes are highly conserved among various organisms. This suggests the acquisition of common ancestral lineages by eukaryotes. In addition, to the similarity in components of trafficking in eukaryotes, each organism has also acquired various specific regulatory molecules which ascertain the diversification to membrane trafficking. In this review we summarize the progress in recent times about the plant-specific Rab GTPases in membrane trafficking events. Rab GTPases are a diverse group which are involved in various processes of membrane trafficking. Further, there are some reports which suggest Rab GTPases' role in signalling pathways involving light, hormones, biotic, and abiotic stresses. Despite these there is still some inhibition among the scientific community to ascribe the latter roles to Rab GTPases with certainty even though the membrane trafficking events are integrated with signalling.

Keywords

Arabidopsis thaliana • Effectors • Rab GTPases • Regulators • Signalling

3.1 Introduction

3.1.1 GTPases in Signalling

The GTP-binding proteins are known to regulate several processes such as signalling, organization of cytoskeleton, and trafficking in eukaryotes (Vernoud et al. 2003; Takai et al. 2001; Boguski and McCormick 1993). The cycling of

R. Ul Rehman (✉)
Department of Bioresources, University of Kashmir, Srinagar 190006, Jammu and Kashmir, India
e-mail: reiazrehman@yahoo.co.in

G.-P. Di Sansebastiano
Di.S.Te.B.A., Università del Salento, via prov. Lecce-Monteroni, 73100 Lecce, Italy
e-mail: gp.disansebastiano@unisalento.it

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GTP-binding proteins from activated form (GTP-bound) to inactivated form (GDP-bound) allows them to function as molecular switches. Inactivation of these proteins takes place by GTP hydrolysis to form GDP. This cycling mechanism between active forms to inactive form is said to be ubiquitous in eukaryotic systems. The members of GTPase (GTP-binding protein) class belong to families of signalling proteins. This family of proteins is reported to be evolutionarily conserved, and these proteins possess functional homologues among the different groups of organisms such as plants, yeasts, and humans. Thus, their importance in cell signalling processes cannot be underscored. The GTP hydrolysis in these regulatory proteins is conserved; however, the other domains in these proteins have been reported to be variable which undergo conformational changes during switching from active state to inactive state. The changes in the protein conformations have been advantageous to eukaryotes in regulating numerous cellular processes (Takai et al. 2001). These molecular switches are controlled physiologically through their association with guanine nucleotide exchange factors (GEFs). These GEFs are accessory proteins which are involved in catalyzing the conversion to active conformation of GTP-binding protein (Vernoud et al. 2003). After activation these small GTPases interact with several effector proteins which perform several cellular functions. The inactivation process takes place either intrinsically wherein hydrolysis of GTP to GDP+Pi is performed by GTP-binding protein itself or by attaching with accessory proteins (GTPase-activating proteins: GAPs) which help in stimulating the hydrolysis activity. After GTP hydrolysis the GTP-binding protein returns back to inactive state and becomes ready for other cycle. The GTPase is a large superfamily and based on various structural and functional similarities among the member's five distinctive families have been established. These families include Arf, Ras, Rho, Ran, and Rab (Kahn et al. 1992). The Ras GTPase members are reported in regulating the cell proliferation in mammalian and yeast systems. The Rho GTPases are reported to have the role in cytoskeleton organization such actin reorganization and in MAP kinase-associated signalling pathways. The members of Rab GTPases and Arf GTPases have been shown to function particularly in membrane trafficking steps. The other members such as Ran GTPases (nuclear proteins) and Ras GTPases have been shown to be responsible for protein/RNA transport regulation across the nuclear membrane. The individual members of Arf, Ras, Rho, Ran, and Rab families share high sequence conservation among themselves than with other small GTPase families as reported in yeast (*Saccharomyces cerevisiae, Saccharomyces pombe*), nematode (*Caenorhabditis elegans*), thale cress (*Arabidopsis thaliana*), fruit fly (*Drosophila melanogaster*), and human (*Homo sapiens*). Vernoud et al. (2003) have given description of identity and classification of GTP-binding proteins based on phylogenetic analysis in *Arabidopsis* classifying all proteins within four groups, viz., Rab GTPases, Arf GTPases, Rho GTPases, and Ran GTPases. There are no Ras GTPases in *Arabidopsis* which reflects that plants have unique mechanisms for signal transduction during development (Meyerowitz 1999, 2002).

3.1.2 Arabidopsis Rab GTPase Family

The Rab GTPases is the largest family among the superfamily of small GTP-binding proteins. The roles of the individual GTP-binding proteins in intracellular membrane trafficking have been ascribed by diversity of experiments in vitro as well as in vivo. The Rab GTPases in plants appear to have followed such diversification and evolution that it has been found that there are 57 Rab GTPases in *Arabidopsis thaliana* genome (Table 3.1) (Vernoud et al. 2003; Pereira-Leal and Seabra 2001). These Rab GTPases are classified into 8 groups from RABA-RABH. These groups have been shown to have sequence and functional similarity with the mammalian Rabs (RAB1, 2, 3, 5, 6, 7, 8, 11, and 18) (Vernoud et al. 2003; Rutherford and Moore 2002). These above groups of Rabs have been reported to be present in most land plants. However, there are few plants

Table 3.1 *Arabidopsis thaliana* Rab GTPases: subclasses, homology with mammalian and yeast systems and the probable function in plants

Arabidopsis thaliana Rab GTPases	Subclasses	Counterparts in mammals	Counterparts in yeast (*Saccharomyces cerevisiae*)	Probable function in plants
RabA	RabA1 (a,b,c,d,e,g,h,i)	Rab11 (Rab11a, Rab11b); Rab25	Ypt31/Ypt32, Ypt3 (*S. Pombe*)	Diverse functions due to several groups. Associated with Golgi vesicles and TGN and endosome
	RabA2 (a,b,c,d)			
	RabA3; RabA4 (a,b,c,d,e)			Golgi-PM trafficking also involved in signalling?
	RabA5 (a,b,c,d,e)			
	RabA6 (a,b)			
RabB	RabB1 (a,b,c)	Rab2	Not seen	ER and Golgi apparatus (both anterograde and retrograde)
				ER-Golgi trafficking
RabC	RabC1, RabC2 (a,b)	Rab18	Not seen	Lack of evidence
RabD	RabD1, RabD2 (a,b,c)	Rab1	Ypt1 (also in *S.pombe*)	ER-to-Golgi trafficking
RabE	RabE1 (a,b,c,d,e)	Rab8, Rab10	Sec4, Ypt2 (*S.pombe*)	Post-Golgi transport to the PM
RabF	RabF1, RabF2 (a,b)	Rab5, Rab22	Ypt51/Ypt52/Ypt53, Ypt10, Ypt5 (*S. Pombe*)	Endocytosis and endocytic-sorting pathways
RabG	RabG1, RabG2, RabG3 (a,b,c,d,e,f)	Rab7	Ypt7 (also in *S. pombe*)	Transport to the vacuole/ lysosome osmotic stress
RabH	RabH1a, RabH1e	Rab6	Ypt6,Ryh1 (*S.pombe*)	Golgi to ER stress responses

For details refer to Lycett (2008), Rutherford and Moore (2002), and Vernoud et al. (2003)

where there are additional Rabs, but their functions are yet unknown (Banks et al. 2011). The land plants have one unique distinction in having the expanded group of RABA/RAB11 (Rutherford and Moore 2002). It has been shown in *A. thaliana* that out of 57 Rab GTPases, 26 belong to RABA/RAB11 group. These 26 Rab GTPases of RABA/RAB11 group are further subdivided into six groups: RABA1, RABA2, RABA3, RABA4, RABA5, and RABA6 (Rutherford and Moore 2002). In contrast among the 66 Rab GTPases known in *Homo sapiens,* three belong to Rab11 group, and among the 11 Rab GTPases known in *Saccharomyces cerevisiae,* only two belong to the Rab11 group (Stenmark and Olkkonen 2001; Pereira-Leal and Seabra 2001). The RABA/RAB11 group is diverse in land plants, and this diversity points to the fact the individual members have acquired role in unique functions. The uniqueness in function has been shown with several studies, e.g., in the cell plate formation RABA2 and RABA3 are essential (Chow et al. 2008). Similarly, for tip growth processes in root hairs and pollen tubes, RABA1 and RABA4 are essential (Szumlanski and Nielsen 2009; de Graaf et al. 2005; Preuss et al. 2004). Additionally the members of the plant RABA have been reported to show localization around trans-Golgi network (TGN) (Szumlanski and Nielsen 2009) which is also thought to act as the endosomes in plant cells (Viotti et al. 2010). Indeed the members of Rab11 from yeast and animals have also been shown to function at various steps in post-Golgi trafficking pathways (Strickland and Burgess 2004).

Among the Ras superfamily the Rab GTPase family is the largest, and these are acting as molecular switches which act as the regulators in cellular transport, thus helping in the vesicle targeting and tethering to cellular membranes. These processes take place by conformational changes in Rab GTPases from active state (GTP-bound) to inactive state (GDP-bound) (Saito and Ueda 2009) (Fig. 3.1). The activation reaction

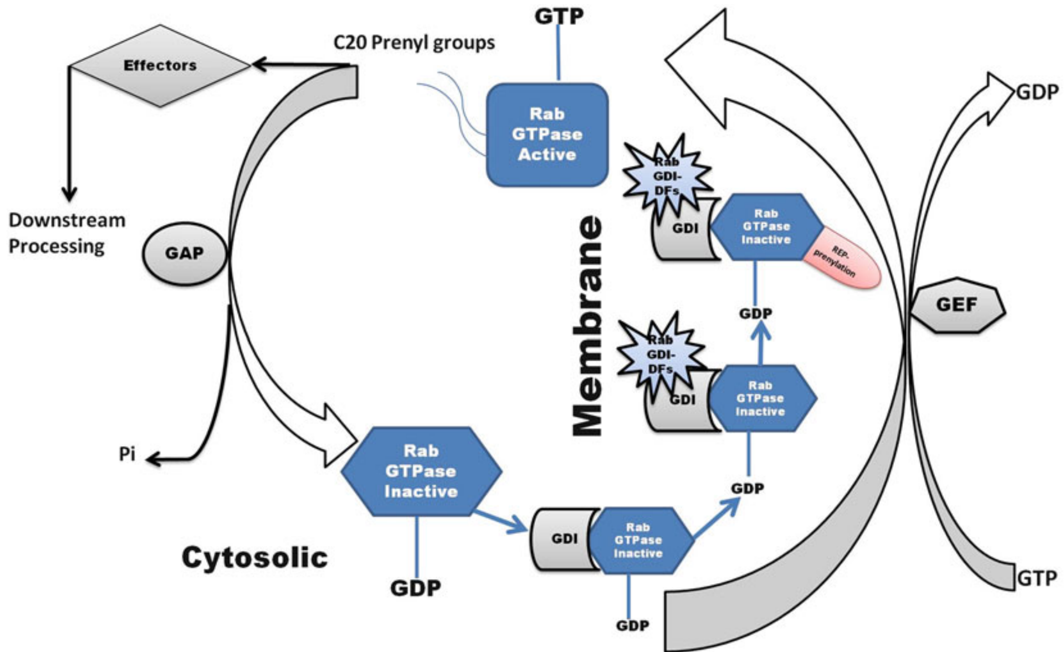

Fig. 3.1 The figure depicts the cycle of Rab GTPases between the cytosolic inactive forms to the membrane-bound active form. The GDP-bound Rab GTPase is cytosolic and exists in Rab GDI complex. This complex is recruited to the membrane by Rab GDI – displacement factors. The Rab escort protein (REP) is required for the prenylation of Rab proteins, and the REP has been known to recognize the newly synthesized Rab protein in the GDP-bound form. Subsequently the specific guanine nucleotide exchange factors (GEFs) are recruited which help in converting the protein to GTP-bound form. This GTP-bound form is insensitive towards the GDI removal at the membrane, and in this form these Rab proteins can recruit the effectors. Finally the GTPase-activating proteins (GAPs) are necessary for the GTPase activity of the Rab protein. After it performs its function, it cycles back to inactive GDP-bound form which is ultimately taken away from the membrane by GDI (Behnia and Munro 2005; Grosshans et al. 2006; Markgraf et al. 2007; Magee and Seabra 2003; Olkkonen and Stenmark 1997; Pereira-Leal and Seabra 2001)

of Rab GTPases (GTP-binding) is catalyzed by guanine exchange factor (GEF). The Rab GTPases are shown to interact with multi-subunit complexes and proteins, and among these interactors some have been shown to act as effectors. The interacting multi-subunit complexes of Rab GTPases include exocyst, HOPS, TRAPP, and the interacting fibrous proteins include EEA1 and P115/USo1p (Markgraf et al. 2007; Cai et al. 2007). The interaction of Rab GTPases with effectors invokes the downstream reactions which help in tethering of vesicles (Transport carriers) to target membranes within the cells (Grosshans et al. 2006). The Rab GTPases have been shown to have higher degrees of phylogenetic diversification (Pereira-Leal and Seabra 2001), and among the class of molecules involved in tethering processes, they are expected to have played important roles in endomembrane system diversification (Elias 2010). The diversification is such that it was suggested based on genomic analysis that the common eukaryotic ancestor might have about 23 groups of Rab GTPases (Elias et al. 2012). This number of groups is even more than found in living eukaryotes including the plants (Fujimoto and Ueda 2012). Thus based on the above findings, it may be mentioned that during the course of evolution, some Rab GTPases were lost and at the same time some Rab GTPases were acquired by eukaryotic organisms (Fujimoto and Ueda 2012) (Table 3.2).

Table 3.2 *Arabidopsis thaliana* Rab GTPase genes

Gene names			AGI gene[a]	Accession no[b]	Expression/localization[c]	References
A	B	C				
AtRABA1a	*AtRab11E*	*Ara-2*[i]	At1g06400	114086		
AtRABA1b			At1g16920	3024526		
AtRABA1c			At5g45750	9758678		
AtRABA1d	*AtRab11B*	*AthSGBP*[ii]	At4g18800	7438436	Highly expressed in roots	Yi and Guerinot (1994)
AtRABA1e			At4g18430	7438435		
AtRABA1f			At5g60860	10176913		
AtRABA1g			At3g15060	8777489		
AtRABA1h			At2g33870	1707014		
AtRABA1i			At1g28550	6560749		
AtRABA2a	*AtRab11C*		At1g09630	3024516		
AtRABA2b			At1g07410	8778562		
AtRABA2c	*AtRab11A*		At3g46830	3915842		
AtRABA2d			At5g59150	9759237		
AtRABA3			At1g01200	9665141		
AtRABA4a			At5g65270	10178189		
AtRABA4b	*AtRab11G*	*AtGB3*[iii]	At4g39990	7438426		
AtRABA4c			At5g47960	9758522		
AtRABA4d			At3g12160	9294115		
AtRABA4e			At2g22390	7438435		
AtRABA5a			At5g47520	9758780		
AtRABA5b			At3g07410	6041856		
AtRABA5c	*AtRab11F*	*Ara-4*[i]	At2g43130	114089	Ubiquitous expression/Golgi-derived vesicles	Anai et al. (1994) and Ueda et al. (1996a)
AtRABA5d			At2g31680	4582469		
AtRABA5e	*AtRab11D*	*Ara-1*[i]	At1g05810	114085		
AtRABA6a			At1g73640	6692728		
AtRABA6b			At1g18200	9719734		
AtRABB1a	*AtRab2B*		At4g17160	7438380		
AtRABB1b	*AtRab2C*	*AtGB2*[iii]	At4g35860	2129702		

(continued)

Table 3.2 (continued)

Gene names						
A	B	C	AGI gene[a]	Accession no[b]	Expression/localization[c]	References
AtRABB1c	*AtRab2A*		At4g17170	1765895	High expression in cotyledons, fruits, and pollen	Moore et al. (1997)
AtRABC1	*AtRab18*		At1g43890	2231312	Stems and roots	Mikami et al. (1998)
AtRABC2a	*AtRab18B*		At5g03530	11274537		
AtRABC2b	*AtRab18C*		At3g09910	6681328		
AtRABD1			At3g11730	4097557		
AtRABD2a	*AtRab1B*	*Ara-5*[i]	At1g02130	5902803	Ubiquitous expression	Anai et al. (1994) and Ueda et al. (1996a)
AtRABD2b	*AtRab1A*		At5g47200	2245111		
AtRABD2c	*AtRab1C*		At4g17530	7268505		
AtRABE1a	*AtRab8B*		At3g53610	11274528		
AtRABE1b	*AtRab8D*		At4g20360	10172744		
AtRABE1c	*AtRab8A*	*Ara-3*[i]	At3g46060	114088		
AtRABE1d	*AtRab8C*		At5g03520	11274535		
AtRABE1e	*AtRab8E*		At3g09900	6681329		
AtRABF1	*AtRab5C*	*Ara-6*[iv]	At3g54840	13160603		
AtRABF2a	*AtRab5A*	*Rha1*[v]	At5g45130	400976	Stomatal guard cells, stipules, and root tips	Terryn et al. (1993)

AtRABF2b	*AtRab5B*	*Ara-7*[iv]	At4g19640	7438427		
AtRABG1			At5g39620	9758338		
AtRABG2	*AtRab7A*		At2g21880	4417298		
AtRABG3a			At4g09720	7438424		
AtRABG3b			At1g22740	3914521		
AtRABG3c	*AtRab7D*		At3g16100	9294457		
AtRABG3d			At1g52280	18389228		
AtRABG3e			At1g49300	5430767		
AtRABG3f	*AtRab7B*		At3g18820	9293907		
AtRABH1a			At5g64990	8843763		
AtRABH1b	*AtRab6A*		At2g44610	7438386	Highest expression in liquid root culture	Bednarek et al. (1994).
AtRABH1c			At4g39890	7438387		
AtRABH1d			At2g22290	4567198		
AtRABH1e			At5g10260	11274352		

Adapted from Nielsen et al. (2008)

[a] AGI gene nomenclature

[b] Genbank protein accession no. (GI) for a translation of the corresponding gene

[c] These are the particular genes which have been seen expressed/localized in different studies. It has its relevance in development i.e. expression of a particular gene at a particular time

A, Nomenclature used in Pereira-Leal and Seabra (2001). B, Nomenclature used in Bischoff et al. (1999). C, Nomenclatures used in: (i) Anai et al. (1991) and Ueda et al. (1996a, b), (ii) Yi and Guerinot (1994), (iii) Biermann et al. (1996), (iv) Ueda et al. (2001), (v) Terryn et al. (1993)

3.2 Membrane Attachment of Rab GTPases

The membrane attachment of Rab GTPases has been reported to be helped by C20 prenyl groups which get attached to two cysteines at the carboxy terminal (Magee and Seabra 2003). The Rab GDP protein is present as Rab GDI (GDP-displacement inhibitor) cytosolic complex, masking the prenyl groups. The recruitment of Rabs to the membrane takes place by the interaction with Rab GDI-displacement factors. Following this, recruitment of specific guanine nucleotide exchange factors (GEFs) takes place which are necessary for converting the protein to GTP-bound form. This GTP-bound form is immune towards GDI removal, and in this active form these Rab proteins can recruit the effectors at the membrane. Finally for the GTPase activity of the Rab protein, the GTPase-activating proteins (GAPs) are necessary. The active form of Rab protein cycles back to inactive GDP-bound form after performing its function and after that the latter taken away from the membrane by GDI ultimately (Markgraf et al. 2007; Grosshans et al. 2006; Behnia and Munro 2005; Magee and Seabra 2003; Olkkonen and Stenmark 1997) (Fig. 3.1).

3.2.1 The Rab Prenylation for Membrane Recruitment

For the prenylation of Rab proteins, the Rab escort protein (REP) is required, and these are known for recognizing the newly synthesized GDP-bound form of Rab protein. The acquiring of GDP-bound form by REP would depend on the GTP hydrolysis of the GTPases (Pereira-Leal and Seabra 2001; Pereira-Leal et al. 2001) (Fig. 3.1). The Rab-REP complex first binds to Rab GGTase followed by the geranylgeranyl-pyrophosphate (GGPP). The later GGPP is reported to increase the affinity of GGTase towards the Rab-REP complex. These have been confirmed with *Arabidopsis* REP which was shown to interact with Rab proteins in GDP-bound form (in tobacco, Arabidopsis, and yeast) and thus stimulating geranylgeranylation in plant extracts (Wojtas et al. 2007; Hala et al. 2005). The phylogenetic analysis reports suggest that REP and GDIs are separate but the REP is related to Rab GDI based on the similarity in structure. This similarity has led to the suggestion that REP and GDIs might have a common evolutionary protein precursor (Hala et al. 2005).

The Yip/PRA1 family members from yeast and mammals have been shown to recruit Rab proteins to the membranes by displacing GDI (Sivars et al. 2003). These membrane proteins are 200 amino acids in length having four transmembrane (TM) domains. Furthermore, certain members of the Yip/PRA1 family are shown to bind with geranylgeranyl groups (Magee and Seabra 2003). Further, it has been shown that by adding one prenyl moiety to soluble Rab protein initiates an interaction with PRA1/Yip3 (Figueroa et al. 2001). There are 19 members in the Arabidopsis PRA1 family grouped into eight clades (AtPRA1.A-AtPRA1H), and most of these proteins form homo and heterodimers (Alvim Kamei et al. 2008).

3.3 Rab-Effector Proteins

There is a diversity in Rab effectors which include tethering factors, enzymes of phosphatidylinositol metabolism, the myosins, the kinesins, and regulators of SNARE protein assembly complex (Markgraf et al. 2007; Grosshans et al. 2006; Behnia and Munro 2005). Many Rab effectors have been characterized in recent years, and these were expected to be conserved and show similar function in all organisms like the Rab GTPases, but this was not the case. Structurally the Rab effectors are seen to be heterogenous which makes it difficult to identify them on sequence similarity only (Zerial and McBride 2001). In *Arabidopsis thaliana* some Rab effectors which belong to lipid kinases have been identified (Christoforidis et al. 1999). Further, the Rab effectors in *Arabidopsis* as well as several other cases have been shown to be associated with zinc finger domains (Heras and Drobak 2002; Jensen et al. 2001; Christoforidis et al. 1999; Simonsen et al. 1998).

3.3.1 Rab-Effector Proteins in Yeast and Animals

In the identification of Rab effectors, significant progress has been made in yeast and animal systems (Pfeffer 2007; Grosshans et al. 2006; Zerial and McBride 2001). The Rab GTPases were earlier reported to function in the regulation of recognition to fusion events on the membranes (Novick and Brennwald 1993; Salminen and Novick 1987). However off late the additional roles have been ascribed to Rab GTPases upon Rab-effector identifications. These new roles in membrane trafficking are vesicle formation, recruitment of motor proteins of cytoskeleton, and tethering and fusion (Hoepfner et al. 2005; Wagner et al. 2002; Carroll et al. 2001; Moyer et al. 2001; Nielsen et al. 1999, 2000; Christoforidis et al. 1999; Guo et al. 1999; Tall et al. 1999; Echard et al. 1998; Wu et al. 1998). The recruitment of Rab effectors and other proteins to the membrane starts with the phosphorylation of phosphatidylinositol (PtdIns) to yield phosphoinositides (PIs), and the phosphorylation reaction takes place in the inositol ring (3,4, and 5 carbon positions). Several PI kinases and phosphatases which are involved in regulation of the PI metabolism are activated by Rab GTPases. The PI 3-kinase (Class III) in mammals has been shown to be an effector of RAB5 and RAB7. These PI 3-kinases on the endosomes phosphorylate PtdIns to PtdIns 3-phosphate (PtdIns(3)P). Further, INPP5B which is known to dephosphorylate PtdIns(4,5)P2 to make PtdIns(4)P has been found to be an effector of many Rab GTPases. The PI 3-kinase (Class I) at the plasma membrane has been shown to be an effector of RAB5, and it phosphorylates PtdIns(4,5)P2 to make PtdIns 3,4,5-trisphosphate (PtdIns(3,4,5)P3). Furthermore the phosphatides PI 5-phosphatase (RAB5 effector) and OCRLI (multiple Rabs effector) at the plasma membrane are involved in dephosphorylation of PtdIns(3,4,5)P3 to PtdIns(3,4)P2. Finally, the PI 4-phosphatase (effector of RAB5) at the plasma membrane is involved in dephosphorylation of PtdIns(3,4)P2 to PtdIns(3)P (Shin et al. 2005; Christoforidis et al. 1999) (Fig. 3.2).

3.3.2 Rab-Effector Proteins in Plants

There is very little information about the Rab effectors in plants (Woollard and Moore 2008), and very few have been identified and characterized. In *A. thaliana* two well-characterized effector proteins are membrane-associated phosphatidylinositol 4-kinases (AtPI4, kβ1, and AtPI4 kβ2). These PI 4-kinases have been shown to interact with RabA4b GTPase to control the trafficking from post-Golgi to plasma membrane in root hair tips (Nielsen et al. 2008). The double mutants of AtPI4 kβ1 and AtPI4 kβ2 genes exhibited reduction in vesicle formation, and it resulted in enlargement of vacuoles and the aberrant growth of root hairs (Preuss et al. 2006). Further, in *Arabidopsis thaliana* the phosphatidylinositol-4-phosphate 5-kinase 2 (PIP5K2) has been shown to interact with all RabE subclass members. This interaction has been proposed to stimulate the production of PtdIns(4,5)P2 (temporally and spatially localized) at the plasma membrane (Camacho et al. 2009). The *Arabidopsis* PtdIns(4) P 5-kinases are involved in various cellular processes such as the apical (tip) growth, stress, and signalling, but their role in regulation of membrane binding is not yet clear (Ischebeck et al. 2008; Kusano et al. 2008; Sousa et al. 2008; Stenzel et al. 2008; Lee et al. 2007; Lou et al. 2007; Mikami et al. 1998). In plants the RabE proteins interact with the type I subfamily B of PtdIns(4)P 5-kinases via the MORN domain which helps in the plasma membrane localization (Camacho et al. 2009). These domains have been shown in Rab5 GEF ALS2 of mammals, and these have a role in GEF activity (Otomo et al. 2003). So, based on these studies it has been proposed that these effectors of Rab GTPases might have a significantly prominent role in organization of endomembrane trafficking (Thole and Nielsen 2008; Žárský et al. 2009). Furthermore, for the Rab-effector function there exist domains for lipid binding which provided the specificity for localization, e.g., the GFP-2xFYVE domains have been shown localized to endosomes indicating the selectivity for PI-3P (Simonsen et al. 1998; Christoforidis et al. 1999; Voigt et al. 2005). As is evident from above, GTP-binding and

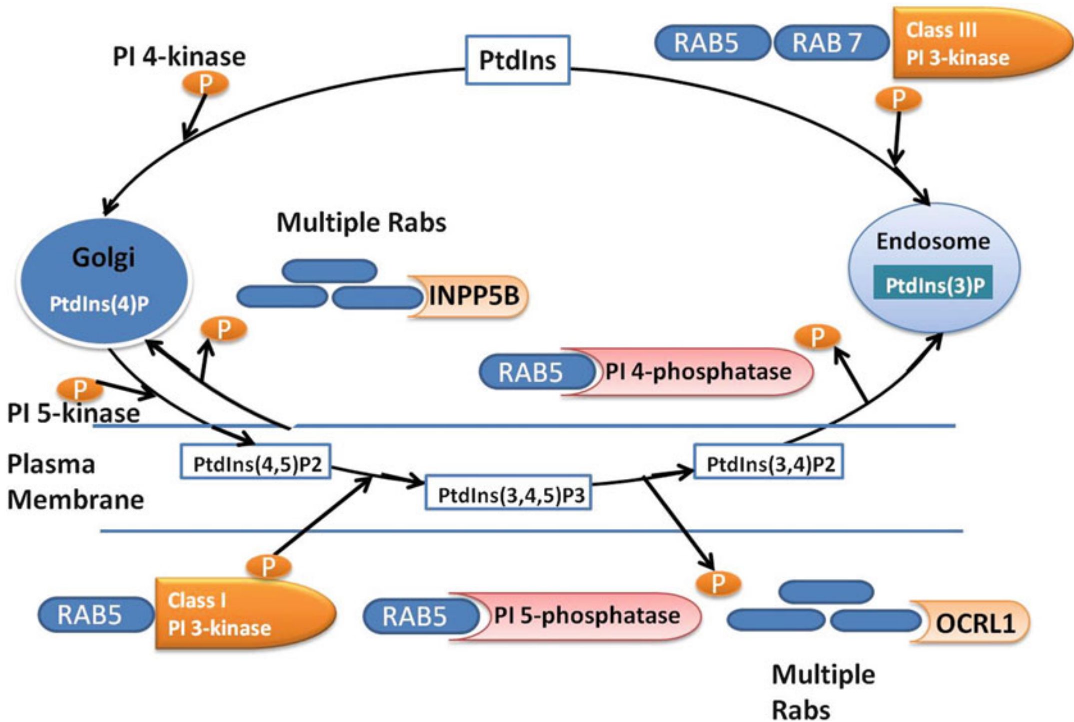

Fig. 3.2 *Phosphoinositides regulation by Rab GTPases.* The recruitment of Rab effectors and other proteins to the membrane starts with the phosphorylation of phosphatidylinositol (PtdIns) to yield phosphoinositides (PIs). In this reaction the hydroxyl groups are phosphorylated at the 3, 4, and 5 carbon positions of the inositol ring. It has been reported that many PI kinases and phosphatases involved in the regulation of PI metabolism are activated by Rab GTPases. In mammals it has been shown that PI 3-kinase (Class III) is an effector of RAB5 and RAB7, and it phosphorylates PtdIns to PtdIns-3-phosphate (PtdIns(3)P) on endosomes. Further, INPP5B has been found to be an effector of multiple Rab GTPases, and it is known to dephosphorylate PtdIns(4,5)P2 to make PtdIns(4)P. Also, PI 3-kinase (Class I) has been shown to be an effector of RAB5, and it phosphorylates PtdIns(4,5)P2 to make PtdIns-3,4,5-trisphosphate (PtdIns(3,4,5)P3) at the plasma membrane. Furthermore the phosphatides PI5-phosphatase which is an RAB5 effector and OCRLI which is effector to multiple Rabs are involved in dephosphorylation of PtdIns(3,4,5)P3 to PtdIns(3,4) P2 at the plasma membrane. Another effector of RAB5 at the plasma membrane is PI 4-phosphatase which is involved in dephosphorylation of PtdIns(3,4)P2 to PtdIns(3)P (Adapted from Stenmark 2009)

hydrolysis are necessary for the regulatory function of Rab GTPases. These studies clearly reveal that Rab GTPases must recruit and then interact with the effector proteins in the cytosol for carrying out the regulatory functional role in membrane trafficking. The GDP-GTP exchange in Rab GTPases is regulatory mechanisms and the two forms (active and inactive) exist in equilibrium, and this balance can be manipulated. It has been shown that the mutations can lead to the formation of constitutively active (CA) or dominant negative (DN) forms which can let the protein in either active or inactive form. The overexpression studies with these CA or DN proteins have helped in understanding the function. Many studies expressing these variant plant Rab GTPases (such as *Arabidopsis* AtRabD2a, AtRabF1, AtRabF2b, AtRab4b, and *Nicotiana tabacum* Rab2) have shown that the Rab regulatory pathway is conserved in eukaryotes (Ueda et al. 2004; Grebe et al. 2003; Batoko et al. 2000).

3.4 Rabs as Regulators of SNAREs

The Rab GTPases are specifically distributed on various cellular membranes, and it has been hypothesized that specific Rab GTPases along with specific SNAREs at a particular location in

the cell provide specificity for membrane fusion (Rehman et al. 2008; Stenmark and Olkkonen 2001; Zerial and McBride 2001), and for this to happen the Rab cycle should be coordinating along with SNAREs (known components of membrane trafficking events). The GTP hydrolysis process enables the syntaxin (a key element in SNARE complex) of the SNARE complex to bind to the vesicle during docking. It has been postulated that during the secretory process which involves a particular SNARE complex comprising of specific syntaxins may eventually coordinate with a specific Rab (Rehman et al. 2008). By transforming the dominant negative mutants of tomato *Le*Rab11 in *Nicotiana tabacum* protoplasts, it was shown that *Le*Rab11 localized in the trans-Golgi network (TGN). The dominant negative (DN) mutant of LeRab11 was used along with specific plasma membrane syntaxins (SYP121 and SYP122). The co-expression of LeRab11 with two plasma membrane syntaxins revealed the appearance of endosomes along with SYP121 and the localization to endoplasmic reticulum along with dotted structures with SYP122. The secretion studies involving secRGUS (Di Sansebastiano et al. 2007) along with the *Le*Rab11 alone, dominant negative *Le*Rab11, and specific syntaxins SYP121 and SYP122 suggested that the Rab11 is regulating the anterograde cellular transport from the trans-Golgi network (TGN) to plasma membrane (PM). This regulation of cellular transport by LeRab11 was shown to be differential involving SYP122 only which suggests that these two individual syntaxins drive different events (Rehman et al. 2008) (Fig. 3.3).

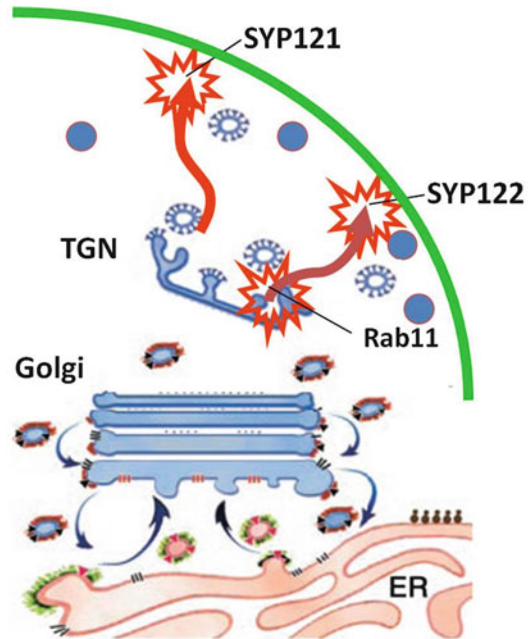

Fig. 3.3 Rab proteins are GTPases cycling between a cytosolic state (GDP-bound) and a membrane-bound active state (GTP-bound). Specific Rab GTPases associate with a particular endomembrane compartment and are involved in specific vesicle transport steps. This figure depicts the role of LeRab11 in vesicle transport. The LeRab11 is localized on the trans-Golgi network and regulates the anterograde traffic to plasma membrane (*PM*). The regulation of cellular transport by Rab11 was shown to be differential involving SYP122 only and not SYP121 which suggests that these two individual syntaxins drive different vesicle transport events (Rehman et al. 2008)

3.5 Rab GTPases in Trafficking Pathways

The Rab GTPases are reported to promote the vesicle (transport carriers) and cytoskeletal interactions (Segev 2001; Zerial and McBride 2001). The members of the Rab GTPase family have distinct roles in vesicle trafficking events (Rutherford and Moore 2002), and sometimes an individual member of the family can perform a role in two distinctive steps in transport pathway (Jedd et al. 1995). The regulatory roles in transport processes by these Rab GTPases are performed by them by interacting with large number of regulatory and effector molecules. The regulatory and effector molecules help in the coupling of GTP binding/hydrolysis to various processes such as vesicle formation, tethering, and docking (Zerial and McBride 2001; Segev 2001).

Another important secretory process is the polarized secretion, and in *Arabidopsis thaliana* the Rab GTPases involved in these processes are the AtRABE subfamily which comprises of five members. These are homologous to mammalian Rab8 and Rab10 (Bischoff et al. 1999) and to *Saccharomyces cerevisiae* Sec4 and *Saccharomyces pombe* Ypt2

(Rutherford and Moore 2002). These proteins involved in transport processes from post Golgi to plasma membrane (PM) may have a role in plant-pathogen interactions (Vernoud et al. 2003) as evidenced by interaction of tomato plant (*Lycopersicon esculentum*) proteins with pathogen (*Pseudomonas* sp.) a virulence protein (avrPto). This study identified a RabE subfamily member similar to mammalian Rab8 (Bogdanove and Martin 2000). This study inferred that in susceptible plants the avrPto might be interfering with membrane trafficking pathway regulated by a RabE member (Vernoud et al. 2003).

3.6 Rab GTPases in Signalling

RabA subclass is the most diversified among the plant Rab GTPases families which might have taken place to fulfil multiple functions in the plants. Among these functions could be the facilitation in the processes of protein distribution of plasma membrane (actin-dependent) to and from internal compartments (Swarup et al. 2001; Geldner et al. 2001; Steinmann et al. 1999). It has been shown that Rab11 and Ypt 32 help in the interaction of actin cytoskeleton to membranes (Lapierre et al. 2001; Ortiz et al. 2002). The motility of organelles in animal cells is microtubule based, while as in plant cells it is actin based. The actin-based motility is said to be complex which might be the reason for RabA subclass diversification in plants (Rutherford and Moore 2002). *Arabidopsis* AtRabA5C (ARA4) has been expressed in yeast ypt mutants to study the interaction with the regulatory factors (Ueda et al. 2000). These overexpression and antisense studies of the members of RabA subclass have resulted in various morphological as well as developmental phenotypes (Lu et al. 2001). In a study involving the pea (*Pisum sativum*) RabA3 (Pra2) protein expression in *Nicotiana tabacum,* tobacco showed the etiolation response. The etiolation response was shown to integrate the brassinosteroid and light signalling pathways (Kang et al. 2001). It was proposed that the Pra2 protein resides on the ER membrane and stimulated DDWF1 which can catalyze a reaction step in brassinolide biosynthesis (Kang et al. 2001). There is a serious discussion about the role of Pra2 in signalling. It has been argued that there is a limited stimulation DDWF1 activity and little interaction in brassinosteroid content in the antisense Pra2 plants and thus cannot be yet concluded with certainty the role of RabA3 sequences is in fact directly involved in brassinosteroid signalling. However, it has been argued on the basis of the localization studies that RabA3 and RabA4 subclass members are acting on transport pathways to vacuoles and the etiolation effect on brassinosteroid metabolism may be an indirect effect by these members leading to defects in vacuole functioning (Schumacher et al. 1999; Inaba et al. 2002; Rutherford and Moore 2002). The role in the growth and orientation of pollen tubes by Rab GTPases thought to be direct by its interaction with the exocyst. However, Ca^{2+} is known for its role in pollen tube orientation and growth, and thus the Ca^{2+} signalling is also thought to be involved in these systems (Lycett 2008). Further the *Arabidopsis thaliana* AtRabA4b interaction with PI-4Kb1(phosphatidylinositol-4-OH kinase) was shown by employing the yeast two hybrid system (Preuss et al. 2006). The *Arabidopsis thaliana* AtRabA4b was also shown to colocalize with PI-4Kb1(phosphatidylinositol-4-OH kinase) in the root hairs. The PI-4Kb1 in the root hairs was shown to interact with *Arabidopsis thaliana* AtCBL1 (calcineurin-like Ca^{2+} sensor). The T-DNA double insertion mutant of PI-4Kb1/PI-4Kb2 showed abnormal root hairs, and upon disturbing the Ca^{2+} gradient by an ionophore A23187 resulted in the RabA4d dispersion from the tip (Preuss et al. 2006). The colocalization study revealed the RabA4 interaction with phosphatidylinositol-4-phosphate phosphatase in the (RHD4) root hair defective four mutants which led to the conclusion that this association may be responsible for regulating the levels of phosphatidylinositol-4-phosphate in root hairs (Thole et al. 2008). These studies make it clear that Ca^{2+} signalling is linked directly to Rab whose action controls the cell wall secretion in tip growing systems (Lycett 2008) (Fig. 3.4).

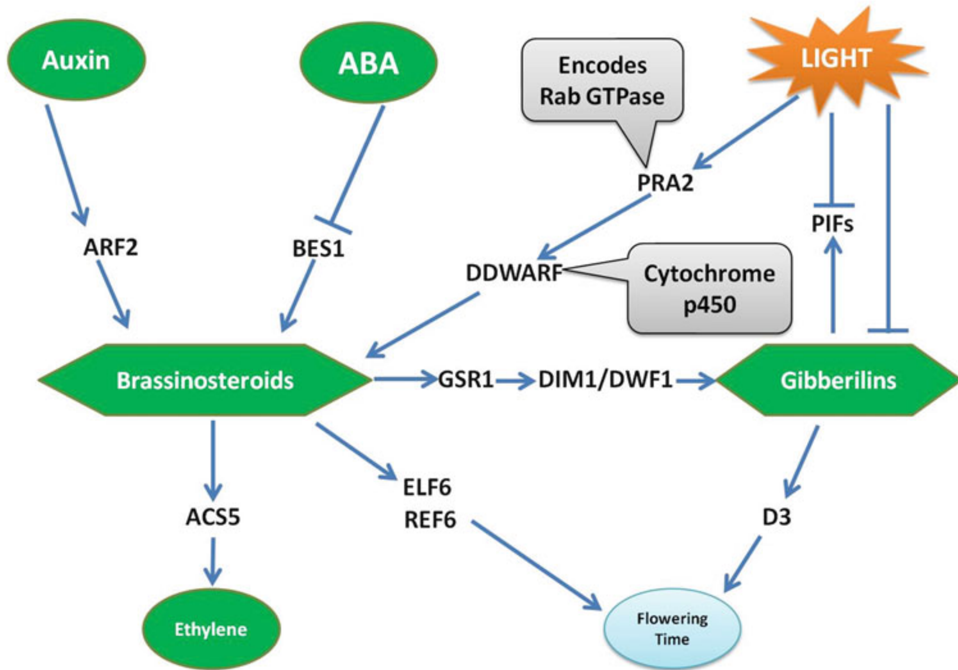

Fig. 3.4 The figure depicts the mechanism of interaction among different phytohormones along with light and flowering time. The figure is the general depiction of different signalling pathways as adapted from Fernandez et al. (2009). The relevance of the figure to the current review is the role of Rab GTPase in brassinosteroid biosynthesis. In *Pisum sativum* a G-protein PRA2 which is regulated by light has been shown to regulate DDWARF. The PRA2 gene encodes the Rab GTPase, while as the DDWARF is a cytochrome P450 which is involved in the oxidation step of the brassinosteroid (*BR*) pathway and thereby integrating the light and the BR signals. The role of BRs in ethylene biosynthesis is stabilizing the 1-aminocyclopropane-1-carboxylate synthase 5 (ACS5). The ACS5 enzyme is involved in synthesis of 1-aminocyclopropane-1-carboxylic acid (ACC) an ethylene precursor. The auxin and BR signalling is integrated by the auxin response factor (ARF2). The ABA signalling has been shown to promote the BES1 phosphorylation and thus inhibit BR signalling. The link between BR and GA pathways is provided by the GAST family gene (as shown in rice OsGSR1) which activates BR biosynthesis DIM1/DWF1 gene. The BR signalling and flowering time control might be linked by two putative histone demethylases ELF6 and REF6 (early flowering 6 and relative of early flowering 6). The light regulates gene expression and cell elongation. The DELLA proteins bind to PIFs (phytochrome interacting factors) transcription factors in the absence of GA and get inhibited. The degradation of DELLA proteins releases the PIFs and allows gene expression. The phytochromes bind to a PIF member and trigger its degradation in presence of light (For detailed review refer to Fernandez et al. 2009) (Source: Adapted from Fernandez et al. 2009)

3.7 Rab GTPases in Cell Wall Biogenesis and Metabolism

The cell wall synthesis and the modification constantly require the cell wall polymers and enzymes. These cell wall components need to be transported or targeted to their location, since the Rab GTPases are known regulators of the vesicle trafficking and thus they have a definite role in the cell wall metabolism (Lycett 2008; Rutherford and Moore 2002). In general biosynthesis of cell wall polymers is known to take place in the Golgi apparatus, but the callose and cellulose are synthesized on the plasma membrane (Lycett 2008). Several plant systems (root tip and pollen tube growth, fruit softening, root nodule development) have been studied for the secretion of cell wall polymers and their modifying enzymes. It has been seen that Rab GTPases are highly expressed in these systems and thus are bound to be

important for the trafficking regulation to the cell wall. Among the Rab GTPases the role of RabA members has been ascertained in this respect. It has been seen that majority of the proteins and enzymes bound for cell wall have signal peptides, i.e., *N*-terminal signal sequences, and their glycosylation occurs in the Golgi (Bayer et al. 2006; Zhu et al. 2006; Boudart et al. 2005; Kwon et al. 2005; Watson et al. 2004; Johnson et al. 2003; Chivasa et al. 2002). These are the strong indications that the protein moment takes place along the endomembrane system (EM) during their synthesis and their transport is bound to take place via the vesicles which are regulated by Rab GTPase (Lycett 2008). The elements of Rab GTPases are shown expressing in fruits, and three Rab1 proteins were identified in tomato (*Lycopersicon esculentum*), and among these LeRab1C is expressed in unripe fruits, and LeRab1A and LeRab1B are expressed in ripe fruits (Abbal et al. 2008; Park et al. 2006; Lu et al. 2001; Zegzouti et al. 1999; Loraine et al. 1996; Zainal et al. 1996). Furthermore, preferential expression of the Rab8 and Rab11 has been shown in tomato (Zegzouti et al. 1999; Lu et al. 2001). The apple fruit has also been seen to possess the same classes of Rabs (Park et al. 2006). Thus the role of Rab GTPases has been suggested in fruit softening by regulating the secretion of cell wall modifying enzymes (Loraine et al. 1996; Zainal et al. 1996). The LeRab11a tomato orthologue silencing resulted in the fruit which remained firm for a longer time period (Lu et al. 2001); however, many developmental and physiological differences were seen in these antisense tomato plants which were said to be due to overexpression of Rab11 GTPases (Kamada et al. 1992; Sano et al. 1994; Aspuria et al. 1995). The abnormal effects were thought to be due to the alteration in trafficking towards the cell wall resulting because of misdirection of protein and hormone carriers/receptors (Lycett 2008; Lu et al. 2001).

Further, in *N. tabacum* leaf protoplasts, the LeRab11a was trans-Golgi network (TGN) associated, and the dominant negative (DN) mutants of LeRab11 were shown inhibiting exocytosis to plasma membrane (PM) (Rehman et al. 2008). In other study on *A. thaliana* root tips, the AtRabA2 and AtRabA3 labelled the cell plate and the dominant negative (DN) mutants disrupted the patterns of cell division indicating their involvement in trafficking to cell plates during the process of mitosis. Further, one dominant negative (DN) mutant targeted periphery in interphase cells, and thus it was inferred that they might also have a role in Golgi to plasma membrane trafficking (Chow et al. 2008). These studies indicate to the presence of several trafficking routes which might be somewhat overlapping and regulated in part by several classes of Rab GTPases (Lycett 2008).

3.8 Role of Rab GTPases in Root Nodules Formation

The process of root nodule formation involves the growth and breakdown of cell wall. The role of Rab GTPases in secretion control in root nodule has been studied in soya bean, and thus Rab7 GTPase and Rab1 GTPase were identified in them (Cheon et al. 1993). Further in the nodules of *Lotus japonicus,* 29Rab cDNAs were isolated, and these comprised of seven copies of Rab1 (role in ER-Golgi transport), five copies of Rab8, and ten copies of Rab11 (role in Golgi/TGN secretion to apoplast) (Borg et al. 1997; Rutherford and Moore 2002; Vernoud et al. 2003). Further, in the nodules of *Medicago sativa*, Rab11f was shown to be expressing (Schiene et al. 2004), and other Rab11 members were shown to be involved in nodule formation by a specific rhizobia (Meschini et al. 2008). The importance of the two Rabs, viz., Rab1 and Rab7, in the nodulation process has been shown by reverse genetic studies, and it was shown that antisense Rab 1 and Rab7 resulted in reduced size of nodule and nitrogenase activity. The antisense expression of Rab1 resulted in reduction of bacteroids, release of bacteroids, and cell expansion. The antisense expression of Rab7 resulted in the suppression of nodule which showed perinuclear accumulation of late endosomes and multivesicular bodies (Cheon et al. 1993).

3.9 Rab GTPases in Tip Growth

The importance of Rab GTPases in tip growth has also been investigated in root hairs and pollen. In *A. thaliana* root hairs, the AtRabF1 and AtRabF2 are associated to endosomes (Voigt et al. 2005), and in pollen the AtRabB1c (Rab2B) was associated with ER-Golgi (Moore et al. 1997). The *N. tabacum* gene NtRab2 of the same class expressed in both pollen and root hairs and also the tissues secreting enzymes or other materials to the cell wall (Cheung et al. 2002). The NtRab2 dominant negative (DN) prevented protein and invertase enzyme trafficking to the cell surface and inhibiting the pollen tube growth. These results show the importance of Rab GTPases in trafficking of material for tip growth (Lycett 2008). Further, the role of Rab11/RabA class in these processes has also been characterized. It has been shown that the *Medicago truncatula* MtRab11G and *A. thaliana* AtRabA4b are expressing in the root hairs (Preuss et al. 2004; Covitz et al. 1998, 2006). The AtRabA5c showed expression in pollen tubes and localized on Golgi cisternae, and vesicles derived from trans-Golgi network (TGN) and Golgi (Ueda et al. 1996a, b). The NtRab11b localized on the cone-shaped region (rich in vesicles) in tobacco pollen tube tips and the NtRab11b dominant-negative mutant (DN) expression led to reduced growth and fertility of pollen tubes (de Graaf et al. 2005) which confirm that the above GTPase is the key element for material delivery to the growing cells. This GTPase also has a role in pollen tube orientation as the dominant negative mutation resulted in pollen tube deformations. These phenotypic effects in *N. tabacum* pollen tubes resulted due to the inhibition of endo/exocytic vesicle targeting to tip cone region as well as the inhibition in delivery of apoplastic cell wall proteins. However, the phenotypes in *N. tabacum* studies were not comparable to T-DNA knockout studies of several *A. thaliana* Rab11/RabA GTPases (de Graaf et al. 2005) which may be due to functional redundancy among this class as ten RabA genes have been shown expressing in pollen (Pina et al. 2005). The Rab8 is also implicated in transport from Golgi-plasma membrane (PM), but its involvement in pollen tubes remains to be determined (Lycett 2008).

3.10 Rab GTPases in Abiotic and Biotic Stresses

Plants are affected by the changes in the environment and the attack by the pathogens. The plant system responds to these by adaptation by bringing about the changes at the physiological as well as the biochemical levels. Even after the high degree of adaptability shown by the plants towards these adversities, there is a tremendous loss in productivity and yield all over the world. The understandings of the plant response which help them to adapt against the different stress are important. The studies and the understanding of the molecular processes which are helping in the regulation of the metabolic changes taking place during stress regimes would enable the scientists to develop stress tolerant plants. The adaptations to stress inside the plant cell might require the recycling, removal, and replacing of proteins or molecules from one compartment to another (Agarwal et al. 2009). The cell organelles are bound to endomembrane system and are responsible for biosynthesis of proteins, lipids, and polysaccharides. It has a role as in cell wall biogenesis; it is important for controlling development and in abiotic and biotic stresses (Surpin and Raikhel 2004). The vesicles and the endomembrane vesicles play a very important role in protein transport to and from organelles.

The connection between abiotic stress signalling and endocytosis is not thoroughly studied, but the role of endocytosis in regulation of abiotic stress responses has been reported (Vieria et al. 1996). The involvement of *N. tabacum* NtSyp1, a vesicle trafficking component, is shown as ABA-dependent secretion (Leyman et al. 1999a, b, 2000) and nonhost pathogen response, cell wall deposition (Assaad et al. 2004), and also pathogen-related processes (Nühse et al. 2003).

The connection between the endocytic and the stress signalling processes is reported in animal cells wherein it has been shown that GDI:Rab5 acts as regulator (Cavalli et al. 2001). The signal transduction pathways modulate the cellular physiology to environmental changes, and the GTP-binding protein expression during these stresses may be constitutive or tissue specific (Agarwal et al. 2009). However, it was found that the *Oryza sativa* OsRab7 gene was expressed in root, leaf, and stem (Nahm et al. 2003). It was shown that *A. thaliana* AtRabG3e was expressed in all plant parts during development (Mazel et al. 2004). The environmental stresses have shown to differentially regulate the Rab genes, and as such the Rab2 transcript increased during deficit in relative water content (RWC) in *Sporobolus stapfianus* (a desiccation – tolerant plant). The desiccation as well as rehydration was also shown to increase the Rab2 transcript in *S. stapfianus* roots and *S. pyramidalis* leaves which indicates the activation of the endomembrane system and its role in the protection and repair upon desiccation (O'Mahony and Oliver 1999). This notion is strengthened by the report in *S. stapfianus* subjected to water stress (dehydration/rehydration) showing the accumulation of lipids (phosphatidylcholine and phosphatidylethanolamine) important for synthesis of membrane (Quartacci et al. 1997). During the salt stress a Rab GTPase belonging the Rab5 family was shown to express in *M. crystallinum* (Bolte et al. 2000).

Among the eukaryotic Rab GTPases, the F1 subclass Rab GTPases are unique in a way that their association to the membrane takes place by myristoylation/palmitoylation at the amino-terminal rather than the geranylgeranylation at the carboxy terminal (Rutherford and Moore 2002; Ueda et al. 2001). The RabF1 GTPases have been identified in rice (*O. sativa*), lotus (*Lotus japonicus*) (Borg et al. 1997), and *M. crystallinum*. The RabF1 GTPase induction is shown by salt stress in the halophyte *M. crystallinum* (Bolte et al. 2000).

The inactive GDP-bound Rab GTPase is cytosolic, and the active GTP-bound Rab GTPase is membrane associated. The association to the membrane is promoted by lipid modification in Rab GTPases which takes place posttranslationally. The Rab GDP-dissociation inhibitor protein (Rab GDI) provides the stabilization to Rab GTPases in cytosol, and in *A. thaliana* three Rab GDIs (At RabGDI1-AtRabGDI3) have been identified. The *O. sativa* OsRab7 was also shown to accumulate upon cold, salt, and dehydration stresses. The application of ABA exogenously also helped in accumulation of the OsRab7 transcript (Nahm et al. 2003). The combined treatment of superoxide and salicylic acid was shown to induce *A. thaliana* Rab7 gene (Mazel et al. 2004; Gorvin and Levine 2000). Similarly, upon salt, cold, and dehydration stresses, there was an accumulation of *Pennisetum glaucum* Rab7 gene (Agarwal et al. 2008). All these reports of accumulation of Rab transcripts upon subjecting the plant to different stresses suggest the involvement of Rabs in adaptation responses. The constitutive overexpression of *A. thaliana* AtRab7 increased the osmotic and salt stress tolerance as well as the accumulation of reactive oxygen species (ROS) during the salt stress (Mazel et al. 2004). The increased tolerance to dehydration and salt stress was also shown by *N. tabacum* expressing *P. glaucum* PgRab7 gene (Agarwal et al. 2008). The overexpressing of AtRab7 in *A. thaliana* plants resulted in increased sodium content in shoots, and Na^+ ion accumulation in vacuoles reduced their toxicity (Mazel et al. 2004). The sodium homeostasis regulation by Rab7 is not well understood and needs further studies.

Further, the role of Rab GTPases in biotic stress resistance has been shown by several studies. The DDWF1 activity is regulated by PRA2 (Kang et al. 2001) and in *N. tabacum* there was induction of DDWF1orthologs by phytopathogen inoculation or treatment with a fungal elicitor (Ralston et al. 2001; Czernic et al. 1996). Furthermore, the rgp1 (rice rab-related gene) overexpression studies showed the relationship between pathogen and the wound-induced transduction system (Sano et al. 1994) which otherwise functions independently in the normal plants (Agarwal et al. 2009). The over-expressing rgp1 rice lines had different wound

Table 3.3 Functions of Rab genes in plants

Name of the gene	Plant origin	Function	References
AtRab1b (AtRabD2a)	*A. thaliana*	ER/Golgi protein transport	Batoko et al. (2000)
At-RAB2 (AtRabB1c)	*A. thaliana*	Pollen development	Moore et al. (1997)
ARA6 (AtRabF1)	*A. thaliana*	Membrane fusion at endosome	Ueda et al. (2001)
ARA7 (AtRabF2b)	*A. thaliana*	Vacuole trafficking	Kotzer et al. (2004)
RHA1 (AtRabF2a)	*A. thaliana*	Vacuole trafficking	Sohn et al. (2003)
A.t.RAB6 (AtRabH1b)	*A. thaliana*	Vacuole trafficking	Bednarek et al. (1994)
AtRab7 (AtRabG3e)	*A. thaliana*	Abiotic stress tolerance	Mazel et al. (2004)
RABE1d (AtRabE1d)	*A. thaliana*	Membrane traffic to plasma membrane	Zheng et al. (2005)
RabA4b (AtRabA4b)	*A. thaliana*	Polarized growth of root hair cells	Preuss et al. (2004, 2006)
NtRab2	*N. tabacum*	Pollen development	Cheung et al. (2002)
NtRab11b	*N. tabacum*	Tip-focused pollen tube growth	de Graaf et al. (2005)
LeRab11a	*N. tabacum*	Fruit maturation	Lu et al. (2001)
OsRab7	*O. sativa*	Abiotic stress tolerance	Nahm et al. (2003)
OsRab11	*O. sativa*	Vacuole trafficking	Heo et al. (2005)
rgp1	*O. sativa*	Hormone regulation, pathogen- and wound-induced signalling	Sano et al. (1994)
PRA2	*P. sativum*	Brassinosteroid biosynthesis	Kang et al. (2001)
		Vesicle transport	Matsuda et al. (2000)
PRA3	*P. sativum*	Vesicle transport	Inaba et al. (2002)
PpRab1	*Maritime pine*	Embryogenesis	Gonçalves et al. (2007)
srab1	*Soybean*	Root nodule formation	Cheon et al. (1993)
SsRab2	*Sporobolus stapfianus*	Involved during dehydration tolerance	O'Mahony and Oliver (1999)
Mcrab5b	*Mesembryanthemum crystallinum*	Increased expression in salt stress	Bolte et al. (2000)
		Vacuole trafficking	Bolte et al. (2004)
PgRab7	*Pennisetum glaucum*	Abiotic stress tolerance	Agarwal et al. (2008)
vrab7	*Vigna aconitifolia*	PBM formation in root nodule	Cheon et al. (1993)

response than the control plants by showing increased tillering and reduced apical dominance. These features were ascribed to the increase in the levels of cytokinin in these plants but which thus resulted in an increase in acidic pathogenesis-related proteins. Upon wounding these plants showed the increase in the inducers (salicylic acid/salicylic acid β-glucoside) of acidic pathogenesis-related proteins. Further the increase in SA results in suppression of mRNA level of proteinase inhibitor II which in turn increases tobacco mosaic virus (TMV) resistance (Sano et al. 1994; Kang et al. 1994). These studies strongly infer about Rab GTPases interfering with signalling pathways, and possibility of strong cross signalling between wound and cytokinin biosynthesis pathways cannot be ruled out (Agarwal et al. 2009) (Table 3.3).

3.11 Future Prospects

The further identification of plant Rab-effector proteins is needed urgently for understanding the functions of Rab GTPases during the membrane trafficking events. There are some signalling pathways which have been shown to be conserved among yeast and mammals. However,

the role of Rabs in signal transduction and development in plants is still in infancy. It has been shown that in plants Rabs are involved in specific functions such as hormonal regulation (brassinosteroid biosynthesis), root hair, pollen, nodule development, and biotic and abiotic stress responses. The involvement of Rabs in hormonal signalling is an important area, and detailed studies are needed for searching the missing link between the signal transduction cross talk and the membrane trafficking. Further, the stress responses biotic as well as abiotic are also part of the signal transduction cascade, and the role of Rabs in hormonal signalling is bound to cross talk at some point. The mechanisms of transport of vesicles and repair of endomembranes by Rab proteins need to be investigated during stress conditions. The candidate genes from tolerant plants belonging to the Rab family should be identified and then characterized for developing and engineering stress tolerant plants.

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PtdIns4P and PtdIns(4,5)P_2 as Signalling Phosphoinositides Involved in Tip Growth

4

Laura Saavedra

Abstract

Phosphoinositides (PIs) comprise a family of minor membrane lipids which play important roles in many signal transducing pathways in the cell. The immediate precursor of all PIs is phosphatidylinositol and the sequential phosphorylation of the lipid head group by the action of phosphoinositide kinases results in the generation of seven additional PI species. PI pools serve as spatial pools that in turn are recognized by cytosolic proteins with specific lipid-binding domains, allowing for selective recruitment of these proteins to sites where they then carry out various cellular functions. Signalling through various PIs has been shown to mediate cell growth and proliferation, cytoskeleton organization, vesicle trafficking, regulation of ion channels and nuclear signalling pathways in various eukaryotic models. In this chapter we first discuss about the PI species, how to visualize them in living cells and their specific distribution within the cellular membranes. Second, we focus on the role of PtdIns(4,5)P_2 and its precursor PtdIns4P, in the process of tip growth as mode of example of signalling by PIs in plant cells.

Keywords

Phosphoinositides • Tip growth • PIPK • PI4K • PtdIns4P • PtdIns(4,5)P_2

4.1 Phosphoinositide Species in Eukaryotic Cells

Phosphoinositides (PIs) refer collectively to a group of membrane lipids comprising phosphatidylinositol (PtdIns) and its phosphorylated derivatives, which play central roles in regulating a wide array of physiological processes at intracellular membranes. PIs have a compartment-specific distribution and the fact that they are minor constituents of cell membranes straightaway indicates that these lipids instead of providing a structural role have a signalling role, which is exerted by specifically interacting with a large number of effector proteins (Toker 2002).

PtdIns is the simplest PI. It comprises a glycerol backbone esterified to two fatty acyl chains

L. Saavedra (✉)
Faculdade de Ciências de Lisboa, Universidade de Lisboa, BioFIG, 1749-016 Lisbon, Portugal
e-mail: llborelli@fc.ul.pt

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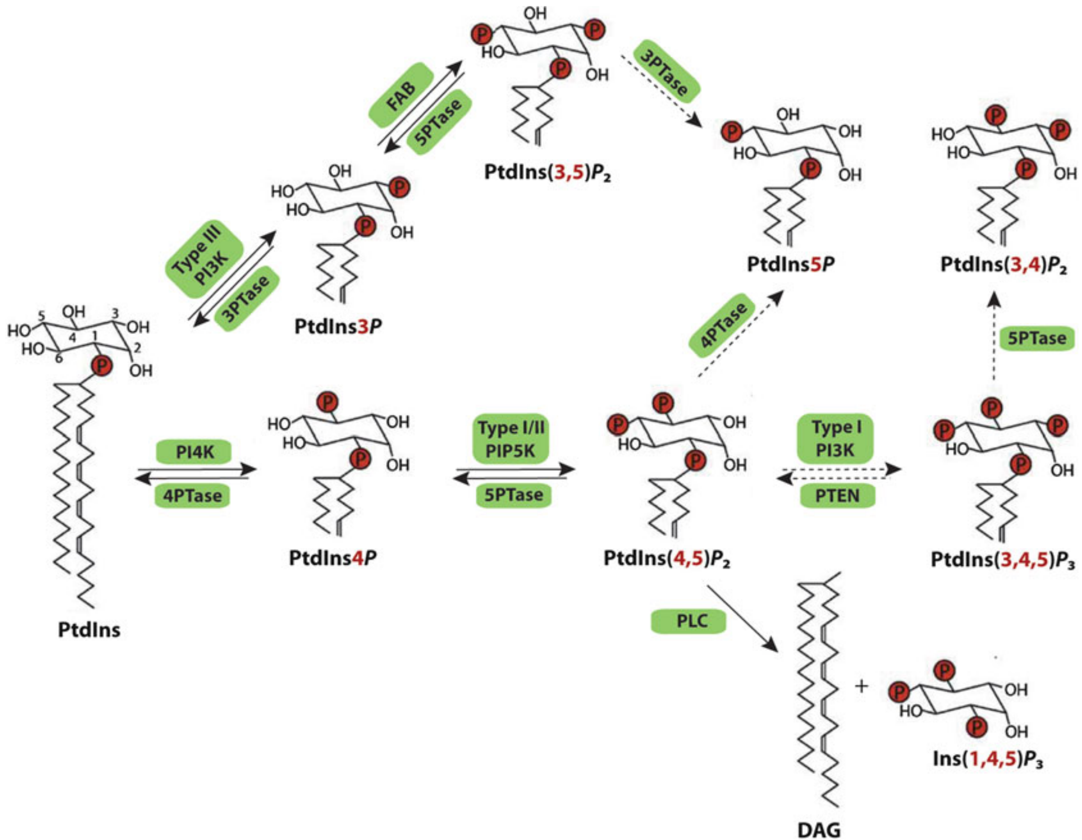

Fig. 4.1 The PI pathway key intermediates: substrates, products and activities of identified phosphoinositide lipid kinases and phosphatases in animal and plant cells. *Solid arrows* indicate that the function of the plant enzyme has been verified. The *dashed arrows* denote that in intact plant cells this reaction has not yet been identified. Some of the acyl chains have been shortened to save space. *Abbreviations*: *PI3K* phosphatidylinositol 3-kinase, *PI4K* phosphatidylinositol 4-kinase, *FAB* phosphatidylinositol 3-phosphate 5-kinase, *PIP5K* phosphatidylinositol phosphate 5-kinase, *PTase* inositol phosphate phosphatase, *PLC* phospholipase C, *DAG* diacylglycerol, *PtdIns* phosphatidylinositol, *PtdIns4P* phosphatidylinositol 4-phosphate, *PtdIns3P* phosphatidylinositol 3-phosphate, *PtdIns5P* phosphatidylinositol 5-phosphate, *PtdIns(4,5)*P_2 phosphatidylinositol (4,5)-bisphosphate, *PtdIns(3,4)*P_2 phosphatidylinositol (3,4)-bisphosphate, *PtdIns(3,5)*P_2 phosphatidylinositol (3,5)-bisphosphate, *PtdIns(3,4,5)*P_3 phosphatidylinositol (3,4,5)-trisphosphate, *Ins(1,4,5)*P_3 inositol (1,4,5)-trisphosphate

and a phosphate to which a cyclic *myo*-inositol head group with five free hydroxyl groups is attached (Fig. 4.1). Of these five hydroxyl groups, positions 3, 4 and 5 are targets for specific PtdIns kinases, while positions 2 and 6 are prevented from phosphorylation, presumably by steric hindrance. There are seven combinations of PIs that can be generated through phosphorylation of one or more positions on the inositol head group: PtdIns3*P*, PtdIns4*P*, PtdIns5*P*, PtdIns(3,4)P_2, PtdIns(3,5)P_2, PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 (Fig. 4.1). Thus far, all of these PI species have been detected in plant cells, with the exception of PtdIns(3,4,5)P_3 (Mueller-Roeber and Pical 2002).

Of all these PIs, PtdIns(4,5)P_2 has far received a lot of attention since the discovery as the main substrate of receptor-stimulated phospholipase C (PLC), by which is hydrolyzed to form two potent intracellular messengers: the water-soluble inositol(1,4,5) trisphosphate (InsP_3) and the membrane-bound diacylglycerol (DAG) (Berridge 1983). In animal cells, InsP_3 promotes the release of calcium from intracellular stores, whereas DAG

promotes the activation of protein kinase C. PtdIns(4,5)P_2 is also recognized as a signalling molecule itself, having diverse roles in cytoskeleton organization (Yin and Janmey 2003), vesicular trafficking (Wenk and De Camilli 2004), secretion (Martin 2001), regulation of ion channels (Suh and Hille 2005) and nuclear signalling pathways (Gonzales and Anderson 2006).

During the last decade, the PI system in plant cells has acquired special interest and intense research has yielded new knowledge in this area. In this chapter, as a mode of example of signalling by phosphoinositides, we will focus on the mechanisms underlying the role of PtdIns(4,5)P_2 and its main precursor, PtdIns4P, in tip-growing cells. Functions of the other PI species will not be addressed here, and we therefore refer the reader to excellent reviews on the topic (Boss and Im 2012; Delage et al. 2013; Heilmann 2009; Mueller-Roeber and Pical 2002; Munnik and Nielsen 2011; Thole and Nielsen 2008; Xue et al. 2009).

4.2 Imaging Phosphoinositides in Live Cells

Both PIs and PI-binding proteins have been shown to exhibit a restricted, rather than a uniform, distribution across intracellular membranes. Therefore, PI lipid pools can serve as spatial pools that in turn are recognized by cytosolic proteins with specific lipid-binding domains, allowing for selective recruitment of these proteins to sites where they then carry out various cellular functions (Thole and Nielsen 2008). In general, lipid-binding domains employ specific lipid head-group recognition complemented by additional hydrogen bonding and nonspecific electrostatic interactions to reversibly associate with the membrane (Scott et al. 2012). In plant cells, at least seven conserved PI-binding domains have been identified to date, including PH, FYVE, PX, ENTH, ANTH, TUBBY and FERM (Scott et al. 2012). These lipid-binding domains are of great importance because they provide research tools to in vivo estimate the subcellular distribution of PIs. If a fluorescent protein is coupled to a lipid-binding domain and stably or transiently introduced into a plant cell, one may visualize the spatiotemporal dynamics of PI microdomains in single cells (Balla and Varnai 2002). In the following sections we will refer to some of these lipid-binding domains and describe how their use when fused with fluorescent proteins has revealed new insights about the role of PIs in plant cells.

4.2.1 Pleckstrin Homology Domain

The name pleckstrin homology (PH) refers to a ~120 amino acid region of sequence homology that occurs twice in pleckstrin and in numerous other proteins with membrane-associated functions; ~250 PH domains are present in the human proteome (Lemmon and Ferguson 2000). The amino acids conforming the PH domain are folded in a highly conserved three-dimensional structure despite little sequence similarity between the family members. This high sequence variability confers to the PH domain's diverse functions interacting with proteins, acidic phospholipids, inositol polyphosphates and PIs (Kutateladze 2010). The PI-binding site of the PH domain contains a cluster of basic lysine and arginine residues which make direct contact with the PI- phosphate groups giving an electrostatically polarized PH domain with a strong positive electrostatic potential around the binding site providing both specific PI-binding and nonspecific electrostatic interactions with other anionic lipids in membranes (Kutateladze 2010).

The PLCδ1 PH domain (PLCδ–PH) binds specifically to PtdIns(4,5)P_2 (Varnai and Balla 1998). Several PH domains have been described in plants (Deak et al. 1999; Jin et al. 2001; Mikami et al. 2000; Stevenson et al. 1998; Tang et al. 2005) and ~50 PH-containing proteins are encoded by the *A. thaliana* genome (Meijer and Munnik 2003). The fluorescence-tagged PH domain of human PLCδ1 has been used to visualize the distribution of PtdIns(4,5)P_2 in plant cells. For instance, in tip-growing cells, such as pollen tubes and root hairs, PtdIns(4,5)P_2 was shown to localize in the tip of these cells at the plasma membrane (Dowd et al. 2006; Helling

et al. 2006; Ischebeck et al. 2008, 2010; Kost et al. 1999; Vincent et al. 2005; Zhao et al. 2010), and its distribution is different depending on the state of the cell. In growing pollen tubes the fluorescence signal is localized in the tip of the growing tube, whereas in nongrowing tubes the labelling is uniform throughout the pollen tube (Dowd et al. 2006). Similarly, the accumulation of PtdIns(4,5)P_2 in the plasma membrane and clathrin-coated vesicles in response to salt stress (Konig et al. 2008; van Leeuwen et al. 2007), its presence during the final stage of a BY-2 cell division (van Leeuwen et al. 2007) and its increase at the plasma membrane upon illumination of guard cells (Lee et al. 2007) have been detected.

4.2.2 FYVE Domain

FYVE is an abbreviation of the first four proteins in which this domain was first identified (**F**ab1p, **Y**OTB, **V**ac1p, **E**EA1). The FYVE domain specifically binds to PtdIns3*P* primarily found in early endosomes, multivesicular bodies (MVB) and phagosomes. FYVE domains are highly conserved from yeast to mammals and plants; there are 27 human (Kutateladze 2012) and 15 *A. thaliana* proteins (Wywial and Singh 2010) containing this domain. The FYVE domain consists of a ~70 amino acid zinc-binding finger which is defined by the three conserved sequences: the WxxD at the N-terminal end, the RR/KHHCR followed by the RVC motifs towards the C-terminus (Kutateladze 2012). Of these, the RR/KHHCR basic motif surrounding the third zinc-co-ordinating cysteine residue distinguishes the FYVE finger family from other structurally related zinc-fingers and is essential for the binding of PtdIns3*P* (Jensen et al. 2001).

A FYVE-GFP construct has been used to visualize PtdIns3*P* in the internal membranes in mammalian cells, labelling endosomes and Golgi and MVB, consistent with the role of PtdIns3*P* in endocytosis and membrane trafficking (Gillooly et al. 2001). In a comparable manner, the FYVE domain has been used to visualize PtdIns3*P* in *A. thaliana* (Jung et al. 2002; Kim et al. 2001; Lee et al. 2008; Park et al. 2003; Vermeer et al. 2006) and in soybean cells (Kale et al. 2010). Using a YFP:2xFYVE as PtdIns3*P*-specific biosensor, *A. thaliana* plants and suspension-cultured tobacco BY2 cells stably expressing lines revealed that the YFP fluorescence signal strongly colocalized with the late endosomal/prevacuolar marker, AtRABF2b, and partially colocalized with the endocytic pathway marker FM4-64 (Vermeer et al. 2006). In plants, PtdIns3*P* is produced by PtdIns 3-kinases encoded by a single copy gene (*VPS34*) in the *A. thaliana* genome (Mueller-Roeber and Pical 2002). *AtPI3K* is expressed in almost all vegetative tissues including root hairs (Lee et al. 2008). Since homozygous *vps34* mutants are lethal, an elegant way which has helped to elucidate the role of PtdIns3*P* in root hairs was overexpressing a GFP:2xFYVE construct in *A. thaliana* and therefore blocking the signal transduction downstream of PtdIns3*P* (Lee et al. 2008). As a result, it was shown that PtdIns3*P* has an important role in both vesicle trafficking and ROS formation which are essential processes required for tip growth (Lee et al. 2008). The use of this biosensor allowed also to confirm the hypothesis that the fungal and oomycete effectors with N-terminal RXLR and dEER motifs bound external PtdIns3*P* in order to enter into host plant cells (Kale et al. 2010).

4.2.3 PX Domain

The PX domain, known as well as Phox-homology, is a region of 130 amino acids of sequence homology found in the phagocyte NADPH oxidase (phox) complex (Ponting 1996), which was identified as a PtdIns3*P*-binding domain (Ellson et al. 2002). Although there is very little sequence similarity between the different PX domains, these diverse sequences fold to adopt a common three-dimensional structure with a proline-rich region (PXXP) involved in the interaction with SH3 domains and a group of basic residues shown to coordinate PIs (Kutateladze 2010). Of all PIs, PtdIns3*P* appears to be a primary target of the PX domain-containing proteins as the majority of them are

involved in membrane trafficking, organization of the actin cytoskeleton and protein sorting (Sato et al. 2001). This domain is found in 83 human proteins (Kutateladze 2012) and in *A. thaliana*, 11 genes encode a PX domain (Banerjee et al. 2010). At least two of the *A. thaliana* genes are involved in lipid signalling since they code for PLDζ 1, 2 (Wang 2002). Another gene which binds PtdIns3P through its conserved PX domain is AtSNX2b. AtSNX2b encodes for a sorting nexin and is involved in endosome to vacuolar trafficking (Phan et al. 2008).

4.2.4 FAPP1 Domain

The PH domain of human FAPP1 protein (phosphatidylinositol-four-phosphate adaptor protein-1) has a high affinity for PtdIns4P in vitro (Dowler et al. 2000) and has been used to visualize PtdIns4P in mammalian cells (Balla et al. 2005; Godi et al. 2004). Later, it was used as a PtdIns4P biosensor in plant cells. When the enhanced yellow fluorescent protein (EYFP) fused to the PH domain of FAPP1 was used to transform *A. thaliana*, the fluorescence signal was present in Golgi membranes and at the plasma membrane. Specifically, EYFP–FAPP1 was enriched in the plasma membrane at the tips of growing root hairs of both *A. thaliana* and *M. truncatula* (Thole et al. 2008; Vermeer et al. 2009).

4.3 Phosphoinositide Levels in the Cell

PIs are not uniformly distributed among membranes but appear enriched at different sites, and these defined pools of PIs can be targeted to specific cellular activities (Downes et al. 2005). PI distribution is regulated by the finely tuned balance activity of lipid kinases and phosphatases which are controlled by intracellular signalling cascades. In animal cells, PtdIns comprises approximately 8 % of the total cellular phospholipid content (Kobayashi et al. 1998). Cellular membranes are enriched in various PIs, present at levels of between 0.01 and 5 % of that observed for PtdIns itself (Stephens et al. 2000). PtdIns(4,5)P_2 and PtdIns4P are enriched in the inner leaflet of the plasma membrane and in Golgi membranes, whereas PtdIns3P and its derivative PtdIns(3,5)P_2 confer identity to endosomal membranes (Cullen et al. 2001; De Matteis and Godi 2004; Roth 2004). Accounting for the proportional area of individual membranes, PtdIns3P, PtdIns4P and PtdIns(4,5)P_2 comprise approximately 4–5 % of the total lipid content in the cytosolic face of their host membranes (Roth 2004).

The ratio of PIs in plants differs from that found in animals in that PtdIns$P$ seems to be more abundant than PtdInsP_2, whereas in animals PtdInsP_2 in general is equally or more abundant (Westergren 2001). In plants, the ratio of PtdIns:PtdIns$P$:PtdInsP_2 ranges from 300:17:1 to 10:1:1 and is likely to depend on the cell type, as it is in animal cells (Hetherington and Drobak 1992; Sandelius and Sommarin 1990). Higher plants seem to have lower levels of PtdIns$P$ and PtdInsP_2 than single-cell algae such as *Chlamydomonas* (Munnik et al. 1998). PtdIns$P$s represent less than 1 % of total phospholipids in plant membranes (Meijer and Munnik 2003; Munnik et al. 1998). PtdIns4P is the most abundant isomer, making up approximately 80 % of the plant PtdIns$P$ pool (Meijer et al. 2001; Munnik et al. 1994; Pical et al. 1999), whereas PtdIns5P and PtdIns3P account for approximately 15 and 10 %, respectively, of the ^{32}P-labelled PtdIns$P$ pool in plants (Meijer et al. 2001).

Most of the research on signalling by PIs has been dedicated to phenomena that take place at the cell periphery and the plasma membrane, but from the animal system, it has become clear that there also exists a nuclear PI cycle which is regulated independently from that at the plasma membrane level (Cocco et al. 2009). Nuclear PIs and derived inositol phosphates are now considered essential cofactors for several nuclear processes, comprising DNA repair, transcription regulation and RNA dynamics (Ramazzotti et al. 2010). In plants, the PI cycle enzymes or enzyme activities and their products have also

been detected in the nucleus. For instance, PtdIns3-kinase has been localized to the plant nucleus and nucleolus in *D. carota* cell cultures, having a distribution which coincides with sites of active transcription (Bunney et al. 2000). Another example is a heat stress induction of PtdIns(4,5)P_2, which was found to localize to the plasma membrane but also in the nuclear envelope, nucleolus and punctate cytoplasmic structures (Mishkind et al. 2009).

The PI levels can also vary due to exposure to different stress conditions. For example, the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* accumulate significant quantities of PtdIns(3,5)P_2 following exposure to osmotic-adjusting chemicals (Dove et al. 1997). Mammalian cells synthesize PtdIns(3,4,5)P_3 in response to sucrose-induced osmotic stress (Van der Kaay et al. 1999). In *A. thaliana*, accumulation of PtdIns(4,5)P_2 upon osmotic stress was also demonstrated (DeWald et al. 2001; Konig et al. 2008; Pical et al. 1999). These observations suggest that different eukaryotic organisms utilize unique PI signalling pathways to elicit the necessary cellular adaptations following a change in the osmotic potential of the surrounding environment (DeWald et al. 2001).

In addition, not only the PI levels can vary upon stress but also their fatty-acid composition, which may also be involved in sorting PIs into functionally distinct pools (Konig et al. 2007). It has been shown in *A. thaliana* plants that depending on stress and nonstress conditions, two different pools of PtdIns4*P* and PtdIns(4,5)*P*2 may exist. Under nonstress conditions, species of these two PIs contain mainly saturated fatty acids, whereas upon hyperosmotic stress, transient synthesis of PtdIns4*P* and PtdIns(4,5)*P*2 is enriched in polyunsaturated fatty acids (Konig et al. 2007).

4.4 Phosphoinositide Pathway Intermediates and Enzymes

PtdIns is synthesized in the endoplasmic reticulum (Lofke et al. 2008) from cytidine diphosphodiacylglycerol and D-*myo*-inositol by phosphatidylinositol synthase and is distributed to other subcellular locations by lipid transfer proteins, directed vesicle trafficking, or a combination of both (Phillips et al. 2006). PtdIns can be modified by a variety of specific lipid kinases, phosphatases and phospholipases, generating a family of PI isoforms that comprise a tightly regulated network of distinct lipid pools throughout the cell (Heilmann 2009) (Fig. 4.1).

PtdIns can be phosphorylated to PtdIns4*P* by PtdIns 4-kinase or to PtdIns3*P* by type III PtdIns 3-kinase (Mueller-Roeber and Pical 2002) (Fig. 4.1). A PtdIns 5-kinase enzyme responsible for the synthesis of PtdIns5*P* from PtdIns has not yet been detected in plants, and PtdIns5*P* might thus be derived from the dephosphorylation of PtdIns bisphosphates (Meijer and Munnik 2003) (Fig. 4.1).

4.4.1 PtdIns 4-Kinases: Classification and Modular Structure

PtdIns 4-kinases (PI4K) catalyse the phosphorylation of PtdIns to synthesize PtdIns4*P*, the first committed step towards the synthesis of PtdIns(4,5)P_2, and therefore this step constitutes a potentially crucial point in the regulation of the PI-dependent pathways.

PI4Ks are classified into two major groups; types II and III which differ in size and sensitivity to inhibitors and adenosine. Type II isoforms are strongly inhibited by adenosine and Ca^{2+} (Carpenter and Cantley 1990) and are insensitive to wortmannin (Endemann et al. 1987). They are localized in the cytosol and associated with the Golgi apparatus (Wong et al. 1997). Type III isoforms have a larger size (100–230 kDa), are not inhibited by adenosine and Ca^{2+} and are inhibited by wortmannin. These are membrane-associated proteins (Nakagawa et al. 1996; Wong et al. 1997). Originally, PtdIns 3-kinases were believed to be a type I PI4Ks, but later it was shown that type I enzymes produce PtdIns3*P* (Balla and Balla 2006).

In mammalian cells there are two type II (PI4KIIα and PI4KIIβ) enzymes and two type III (PI4KIIIα and PI4KIIIβ), whereas in yeast, there is one type II enzyme (LSB6P), and two type III

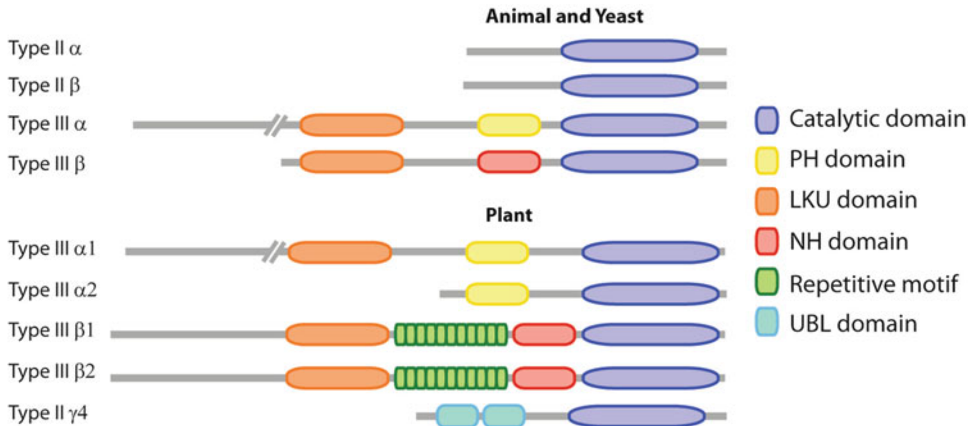

Fig. 4.2 Modular structure of animal, yeast and plant PI4Ks. The various conserved domains are represented by *coloured boxes*. Some plant PI4Ks type IIγ contain one or two ubiquitin-like domains and here is represented only type IIγ4. A truncated version of AtPI4K type IIIα1 is presented due to the large size of the protein and the lack of conserved domains

enzymes (PI4KIIIα known as STT4P and PI4KIIIβ known as IPK1) (Balla and Balla 2006).

Structurally, type III enzymes consist of a catalytic domain of about 230 amino acid residues located at the C-terminal part of the protein and a kinase unique domain (LKU, also known as helical domain) (Fig. 4.2). In PI4KIIIα type, a putative PH domain is located between the helix and catalytic domain, whereas in PI4KIIIβ isoforms a novel homology (NH) domain is located. Type II enzymes have a kinase domain that shows little sequence homology with those of the type III enzymes (Fig. 4.2).

The *Arabidopsis* genome codes for 12 PtdIns 4-kinases in which four belong to the type III group (AtPI4Kα1, AtPI4Kα2, AtPI4Kβ1 and AtPI4Kβ2) and the remaining eight belong to the type II group, also called γ group (Mueller-Roeber and Pical 2002) (Fig. 4.2).

The first genes described for functional PI4K in plants were type III AtPI4Kα1 (Stevenson-Paulik et al. 2003; Stevenson et al. 1998) and AtPI4Kβ1 (Xue et al. 1999). AtPI4Kα1 contains the LKU, PH and catalytic domains. AtPI4Kα2 is a smaller protein, which contains only the catalytic domain lacking the LKU and PH domains, and does not possess PtdIns 4-kinase activity (Stevenson-Paulik et al. 2003). AtPI4Kα1 has been shown to be an active protein in vitro using the baculovirus expression system (Stevenson-Paulik et al. 2003) and its PH domain binds PtdIns4*P* with higher affinity but also binds phosphatidic acid (PA) and PtdIns(4,5)P_2 (Stevenson et al. 1998).

AtPI4Kβ1 and AtPI4Kβ2 share 80 % amino acid identity and consist of the LKU, NH and catalytic domains. Additionally, both proteins exhibit a repetitive motif constituted of 11 repeats of a charged core unit, which is unique for the plant β isoforms (Mueller-Roeber and Pical 2002). AtPI4Kβ1 is an active protein in vitro (Stevenson-Paulik et al. 2003).

Members of the PI4Kγ family consist of a catalytic domain, but in addition six members contain one or two ubiquitin-like domains (UBL) (Mueller-Roeber and Pical 2002). AtPI4Kγ4 and AtPI4Kγ7 have been shown to be protein kinases but none of eight isoforms have yet been demonstrated to have lipid kinase activity (Galvao et al. 2008). It is hypothesized that the plant PI4Kγs are involved in ubiquitin-regulated protein turnover (Galvao et al. 2008).

4.4.2 Role of Type III PI4Kβ in Tip Growth in *A. thaliana*

In plants, PtdIns 4-kinase activity has been detected in different cell locations including the plasma membrane, cytosol and cytoskeleton

(Davis et al. 2007; Drobak 1992; Westergren et al. 1999; Xu et al. 1992; Yang et al. 1993). This wide distribution of PtdIns 4-kinase activity throughout the cell suggests that there are distinct isoforms that are targeted to the various compartments where they perform distinct functions (Stevenson-Paulik et al. 2003).

Tip growth is a mode of cell expansion strictly dependent on polarized exocytosis of vesicles to the apical growth domain, which provide new plasma membrane and cell wall precursors (Lee and Yang 2008). Pollen tubes, root hairs and moss protonemata are examples of cells which follow tip growth. The first functional role for a PtdIns 4-kinase was described by Preuss et al. (2006) where they showed that type III PI4Kβ1 and PI4Kβ2 are essential for trafficking of Golgi-derived vesicles in root hairs. The AtPI4Kβ1 NH domain interacts with AtRABA4B in a GTP-dependent manner and these two proteins colocalize in the tip of growing root hairs. *A. thaliana* T-DNA double *pi4kβ1/pi4kβ2* mutants display root hairs with aberrant morphology which lead the authors propose a model for RABA4B and PI4Kβ1 action during polarized root hair expansion. The same laboratory showed later that AtRABA4D, another member of the *A. thaliana* RabA4 subfamily of Rab GTPase proteins, which is pollen-specific, also interacts with the NH domain of PI4Kβ1 and this interaction is important for proper regulation of polar tip growth in pollen tubes (Szumlanski and Nielsen 2009). Importantly, it is not only the presence of the lipid in this case PtdIns4*P* which is essential but also its turnover. This is exemplified in *A. thaliana* mutants for the *RHD*4/*SAC*7 gene which codes for a PtdIns 4-phosphatase. *rdh4* root hairs are shorter and randomly form bulges along their length, they exhibit double PtdIns4*P* content than wild-type tissue, and a significant amount of PtdIns4*P* is associated with internal membranes, whereas in wild-type root hairs *PtdIns4P* accumulated primarily in a tip-localized plasma membrane domain (Thole et al. 2008). The model proposed that RHD4 is selectively recruited to RabA4b-labelled membranes that are involved in the polarized expansion of root hair cells and together with PI4Kβ1, RHD4 regulates the accumulation of PtdIns4*P* on membrane compartments at the tips of growing root hairs (Thole et al. 2008).

4.4.3 PtdInsP Kinases: Classification, Modular Structure and Biochemical Properties

The following step towards the synthesis of PtdIns(4,5)P_2 is the phosphorylation in the D-5 position of the inositol ring of PtdIns4*P*, by PtdIns4*P* 5-kinases (PIPKs). In animal cells, PIPKs are classified in three different subfamilies (type I–III) according to their substrate specificity, subcellular localization and function (Heck et al. 2007). Types I and II are responsible for the synthesis of PtdIns(4,5)P_2, whereas type III synthesizes PtdIns(3,5)P_2. Type I PIPKs phosphorylate PtdIns4*P* at the D-5 hydroxyl group and therefore are classified as PtdIns4*P* 5-kinases (Rameh et al. 1997; Zhang et al. 1997). Additionally, type I PIPKs can synthesize PtdIns(3,4,5)P_3 by consecutive phosphorylation of the 4- and 5-hydroxyl groups of PtdIns3*P* (Zhang et al. 1997). Type I PIPKs can also produce PtdIns(3,5)P_2 and PtdIns5*P* from PtdIns3*P* and PtdIns, respectively (Tolias et al. 1998). Type II PIPKs are PtdIns5*P* 4-kinases, they synthesize PtdIns(4,5)P_2 by phosphorylation of PtdIns5*P* at the D-4 position. Additionally, they can synthesize PtdIns(3,4)P_2 from PtdIns3*P* (Rameh et al. 1997). The last group, type III PIPKs, represented by the yeast Fab1p enzyme or the mammalian PIKfyve homolog, are PtdIns3*P* 5-kinases which phosphorylate PtdIns3*P* to generate PtdIns(3,5)P_2 (Dove et al. 1997; McEwen et al. 1999).

Animal, yeast and plant PIPKs share a basic structure which consists of a dimerization domain and a highly conserved lipid kinase domain located at the C-terminus (Fig. 4.3) (Mueller-Roeber and Pical 2002). In addition, most plant PIPKs contain the MORN domain (Membrane Occupation and Recognition Nexus) followed by a non-conserved linker region (Fig. 4.3) (Mueller-Roeber and Pical 2002).

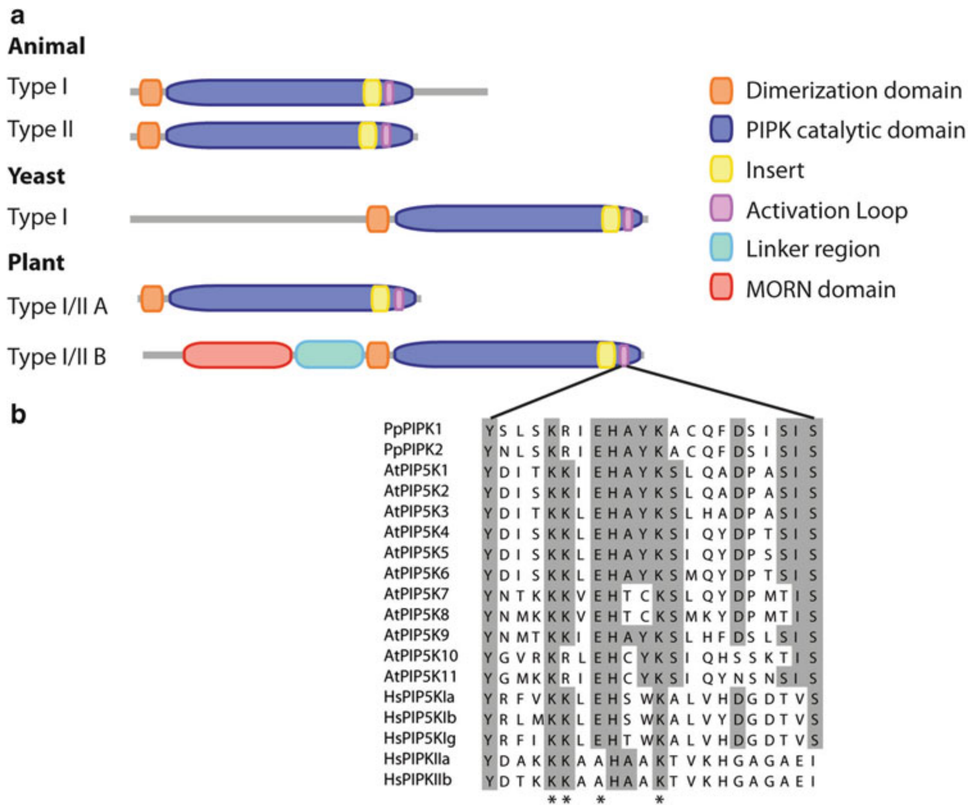

Fig. 4.3 (**a**) Modular structure of animal, yeast and plant type I/II PIPKs. (**b**) Amino acid sequence alignment of the activation loop of *P. patens*, *A. thaliana* and type I and type II *H. sapiens* PIPKs. The *asterisks* indicate conserved amino acids mentioned in this chapter. First, two conserved positively charged amino acids (KR or KK); second, (E or A), which are involved in substrate specificity; third, (K) which is involved in plasma localization of animal type I PIPKs

The MORN domain represents a unique feature of plant PIPKs, it consists of repetitions of MORN motifs and is localized at the N-terminal end of the protein. MORN motifs lacking a PIPK catalytic domain have been found in several animal and plant proteins, such as junctophilins, which participate in endomembrane to plasma membrane attachment (Takeshima et al. 2000); the MORN1 protein of *Toxoplasma gondii* involved in cell division (Gubbels et al. 2006); and the *A. thaliana* accumulation and replication of chloroplasts three protein (ARC3) involved in plastidial fission (Maple et al. 2007).

In the last decade, several PIPK genes from different plant model organisms such as *A. thaliana*, *O. sativa* and *P. patens* have been studied. Based on their amino acid sequence similarity to the corresponding mammalian enzymes, plant PIPKs cannot be assigned to either type I or type II and thus they have been classified as type I/II (Mikami et al. 1998; Mueller-Roeber and Pical 2002). The *A. thaliana* genome contains eleven genes encoding type I/II PIPKs which are grouped into type A and B. Subfamily A consists of two members, AtPIP5K10 and AtPIP5K11, which lack the MORN domain and exhibit a domain structure similar to human type I PIPKs. The other nine remaining isoforms (AtPIP5K1–9) which contain additionally the N-terminal MORN domain belong to subfamily B (Mueller-Roeber and Pical 2002) (Fig. 4.3). In contrast to the high number of PIPK isoforms found in higher plants, the moss *P. patens* encodes for only two genes, *PpPIPK1* and *PpPIPK2*, which belong to the subfamily B and

no members for the A subfamily are present (Saavedra et al. 2009).

Lipid kinase activity assays showed that PtdIns4*P* is the preferred substrate in vitro for the synthesis of PtdIns(4,5)P_2 by all plant PIPKs (Elge et al. 2001; Ischebeck et al. 2008, 2010; Saavedra et al. 2009; Stenzel et al. 2008; Westergren 2001). An exception is for PpPIPK2 which in vitro synthesizes PtdIns3*P* from PI (Saavedra et al. 2009). However, in vivo it was verified that both PpPIPKs synthesizes PtdIns(4,5)P_2, since the phosphoinositide profile for both *pipk1* and *pipk2* single knockout mutants showed a reduction only in PtdIns(4,5)P_2 (Saavedra et al. 2011).

Within the PIPK kinase domain, there is a region, known as the activation loop (AL), containing a conserved glutamic acid residue (Fig. 4.3) which confers substrate specificity to animal type I PIPKs (Kunz et al. 2002). The corresponding glutamic acid residue which is conserved in plant PIPK isoforms has been changed to alanine and Pp*pipk1E885A* or At*pip5k1E715A*-mutated proteins exhibited an almost completely abolished activity towards PtdIns4*P* and PtdIns3*P* in vitro (Saavedra et al. 2009). The relevance of this amino acid in plant PIPKs was also confirmed in vivo since overexpression of *PpPIPK1E885A* could not completely complemented *pipk1* knockout phenotype (Saavedra et al. 2011). In addition, within the activation loop there is a dibasic amino acid pair KR (Fig. 4.3) which is also crucial for the PpPIPK1 lipid kinase activity. The mutation of KR to ND abolished the enzymatic activity towards PtdIns3*P* and PtdIns4*P* in vitro (Mikami et al. 2010).

4.4.4 PIPKs Are Localized to the Plasma Membrane

PIPKs are recruited to membranes but they are not integral membrane proteins. In animal cells it is well established that the AL is also responsible for the differences in subcellular localization between type I and type II PIPKs. A substitution of a conserved glutamic acid located in the AL to alanine results in a swap of substrate specificity and subcellular localization between the two types (Kunz et al. 2000, 2002) (Fig. 4.3). In plants, it was suggested that the MORN domain is responsible for the membrane localization of OsPIPK1, AtPIP5K1 and AtPIP5K3 (Im et al. 2007a; Kusano et al. 2008; Ma et al. 2006). However, data from both PpPIPKs, AtPIP5K1, AtPIP5K2, AtPIP5K5 and NtPIP5K6-1 clearly demonstrated that there are additional modules important for correct subcellular localization (Mikami et al. 2010; Stenzel et al. 2012). For example, the lipid kinase domain is responsible for the membrane localization of PpPIPK1 and AtPIP5K1 (Mikami et al. 2010). Interestingly, AtPIP5K2 kinase domain directs plasma membrane localization but not its apical localization in pollen tubes (Stenzel et al. 2012), suggesting that more than one regulatory component controls apical plasma membrane localization in polarized cells. In contrast, for AtPIP5K5 and NtPIP5K6-1 the non-conserved linker (LIM) domain is required for their correct subcellular localization in pollen tubes (Stenzel et al. 2012). Taking together these results, it is possible that protein modules responsible for plasma membrane localization are distinct in each PIPK depending on differences in physiological and/or developmental status of cells, such as polarized and non-polarized (Saavedra et al. 2012).

4.4.5 Role of PIPKs in Tip-Growing Cells of *A. thaliana* and *P. patens*

As mentioned above, in contrast to what is observed in animal cells, cellular levels of PtdIns4*P* are much higher compared to PtdIns(4,5)P_2 in plants, highlighting a restriction step controlling PtdIns(4,5)P_2 levels by PIPKs and thereby indicating the importance of PIPK regulation in physiological processes requiring PtdIns(4,5)P_2 (Im et al. 2007b). In *A. thaliana* vegetative tissues under normal growth conditions, PtdIns(4,5)P_2 is hard to detect which may account for the scarce information available for such tissues. However, specialized plant cells

such as root hairs, pollen tubes and protonemal cells of mosses (all sharing the process of cell expansion by tip growth) have been used as preferable models for studying PIPK function for several reasons: (1) PtdIns(4,5)P_2 accumulates at the tip of these cells (Ischebeck et al. 2008; Saavedra et al. 2011; Sousa et al. 2008; Stenzel et al. 2008), (2) PtdIns(4,5)P_2 can be followed by using the pleckstrin homology (PH) domain of the human PLCδ1 as a biosensor (Varnai and Balla 1998), and (3) these cell types can be easily studied at a single-cell level.

Detection of PtdIns(4,5)P_2 in membrane microdomains of pollen tubes or in the plasma membrane of root hair cell tips was first reported in 1999 (Braun et al. 1999; Kost et al. 1999). Since then it has been clearly demonstrated that members of the *A. thaliana* and *P. patens* PIPK family play a key role in the process of tip growth. Of the 11 PIPK isoforms present in *A. thaliana*, AtPIP5K3 is specifically expressed in root hairs (Kusano et al. 2008; Stenzel et al. 2008). Root hairs of T-DNA *pip5k3* mutants exhibited reduced growth, and when *AtPIP5K3* was overexpressed in a wild-type background, it resulted in deformed root hairs (Stenzel et al. 2008). Interestingly, a mutated version of AtPIP5K3 lacking the N-terminal MORN domain but with full catalytic activity in vitro could not complement the *pip5k3* mutant phenotype. In addition, when this mutated version of the protein was overexpressed, the resulting phenotype showed deformed root hairs, which implies that AtPIP5K3 functionality in root hair development requires other factors in addition to the catalytic activity (Stenzel et al. 2008).

In contrast to what is observed for root hairs, six PIPK isoforms, AtPIP5K10, AtPIP5K11, AtPIP5K2, AtPIP5K4, AtPIP5K5 and AtPIP5K6, are expressed in pollen tubes. Despite their high sequence similarity, different roles in tip growth have been attributed to them. When fused to a fluorescent protein, it has been shown that both AtPIP5K4 and AtPIP5K5 are expressed at the apical region of the plasma membrane of pollen tubes (Ischebeck et al. 2008; Sousa et al. 2008). The double *pip5k4-pip5k5* mutants exhibited reduced pollen germination and defects in pollen tube elongation (Ischebeck et al. 2008; Sousa et al. 2008). Overexpression of *AtPIP5K4* or *AtPIP5K5* in tobacco pollen tubes led to severe growth defects, which were attributed to increased apical pectin deposition (Ischebeck et al. 2008; Sousa et al. 2008). Another example is AtPIP5K6, which is localized instead in the subapical region of the plasma membrane of pollen tubes, and the suppression of *AtPIP5K6* expression by RNAi resulted in impaired tip growth and inhibited clathrin-dependent endocytosis (Zhao et al. 2010). Different to what is observed for AtPIP5K4, AtPIP5K5 and AtPIP5K6 which belong to the B subfamily, AtPIP5K10 and AtPIP5K11, both members of the A subfamily, were found to localize to the lateral subapical plasma membrane in tobacco pollen tubes. Phenotypes observed for these latter isoforms are remarkably different from those mentioned above. Pollen tubes of *pip5k10-pip5k11* double mutants exhibited increased sensitivity to latrunculin B (an actin polymerization inhibitor), and overexpression of both enzymes in tobacco pollen tubes resulted in aggregation of the apical actin fine structure and a tip-swelling phenotype (Ischebeck et al. 2010). A tip-swelling phenotype was also observed when AtPIP5K2, another type B isoform, was overexpressed (Stenzel et al. 2012). Thus, the mechanisms of action of PtdIns(4,5)P_2 produced by PIPKs are different, some members affect membrane trafficking and secretion, whereas others affect the actin cytoskeleton. It has been suggested that the distinct localization patterns of the enzymes may be the consequence of interactions with specific partner lipids or proteins, which recruit the enzymes to different functional microdomains (Ischebeck et al. 2010). The N-terminal MORN domain, which is present only in type B PIPKs, is not responsible for the different phenotypes observed between type A and B subfamilies; overexpression of *AtPIP5K3* or *AtPIP5K5* mutated isoforms lacking the MORN domain resulted in the same phenotypes as that with the full-length proteins (Ischebeck et al. 2008; Stenzel et al. 2008). Therefore, the results discussed above verify more clear the notion that there are different pools of the same PI even the same cell and that the different pools are functionally and spatially

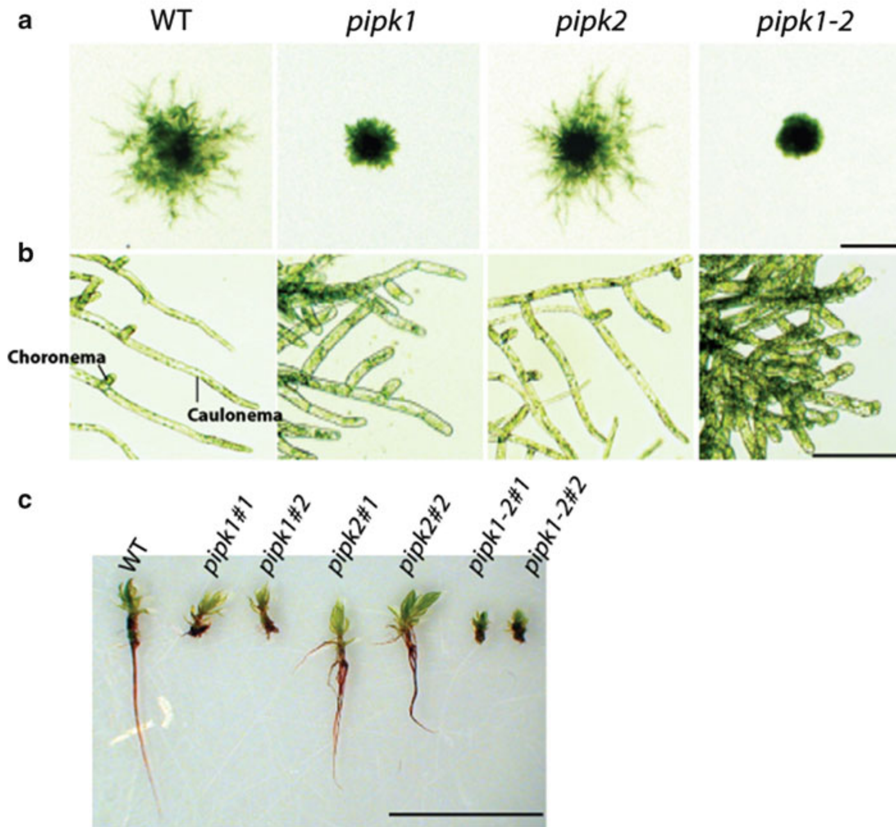

Fig. 4.4 Comparison of the phenotype of *P. patens* wild type and of the single and double *pipk* knockout mutants at different developmental stages (Saavedra et al. 2011). (**a**) Three-week-old colonies growing in minimal media, bar = 0.5 cm. (**b**) Six-day-old protonema filaments growing on minimal media, bar = 200 μm. (**c**) Twenty-day-old gametophores, bar = 0.5 cm

distinct and are influenced by differentially targeted PIPK isoforms.

In *P. patens*, both PIPK genes are expressed in the juvenile protonema and adult gametophytes under normal growth conditions (Saavedra et al. 2009). The disruption of both genes by gene targeting allows to demonstrate that PpPIPKs have also a role in tip growth as observed in higher plants (Saavedra et al. 2011). Despite the high similarity between both proteins, a strong phenotype for *pipk1* but not for *pipk2* single knockouts was obtained, even though PtdIns(4,5)P_2 levels in both single mutants were reduced to half in comparison to the wild type (Saavedra et al. 2011). The *pipk1* knockout lines showed a dramatic growth reduction of rhizoids as well as of protonema, and caulonema cells had a half reduction of their length (Fig. 4.4). A stronger phenotype compared to *pipk1* was then obtained for *pipk1-2* double knockouts, whose PtdIns(4,5)P_2 levels were almost not detected. *pipk1-2* protonemal filaments exhibited an extremely compact structure and lacked the caulonemal cell type; gametophytes were much shorter than the wild type with very short rhizoids and could not produce sporophytes (Saavedra et al. 2011). When the wild-type moss was treated with F-actin destabilizing drugs such as latrunculin B or cytochalasin B, it resulted in a phenotype that mimicked the *pipk* knockout phenotype, suggesting a role of PtdIns(4,5)P_2 in the cytoskeleton organization. This role was confirmed by in vivo imaging of the cytoskeleton network, which revealed

that the shortened caulonemal cells in the *pipk1* mutant were the result of the absence of the apicobasal gradient of cortical F-actin cables normally observed in wild-type caulonemal cells (Saavedra et al. 2011). Therefore, the role of PtdIns (4,5) P_2 in tip-growing cells seems to have been conserved during evolution.

4.5 Conclusions and Future Perspective

During the last years, research about the plant phosphoinositide signal transduction pathway has developed significantly. PI-metabolism is emerging as an exciting area due to the wide spectrum of processes they are involved such as tip growth, abiotic and biotic stress, hormone signalling and energy signalling within others. However, our knowledge about the role of the different PI species in plants is still limited. For instance, for several PI-kinases and phosphatases, there are no functional studies described and very few reports are described in PI-interacting partners which would help to elucidate the downstream signalling pathways which are initiated by PIs. The availability of better genetic tools as well as the use of different evolved plant model systems, together with more sensitive analytical techniques to measure PIs, will provide further insights into the plant PI signalling and metabolism.

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Sugar Signaling in Plant Growth and Development

5

Waseem Shahri, Syed Sabhi Ahmad,
and Inayatullah Tahir

Abstract

Sugars are the primary energy sources produced by green plants via the life-sustaining process of photosynthesis. The metabolic role of sugars as energy compounds and essential metabolites in living organisms has long been recognized. However, genetic and molecular (mutational) studies during the last decade have highlighted the role of sugars as signaling molecules in controlling diverse aspects of plant growth and development. The review focuses on specific signaling roles of various sugars particularly hexoses (glucose and fructose), sucrose, trehalose, and small glycans. Moreover, the sugar-specific regulations of various genes and the diverse signaling cascades involved have been discussed. The role of hexokinase–kinase-dependent and hexokinase-independent signals (like G proteins) in sugar signal transduction pathways has also been documented. The evidences generated from the analyses of sugar-insensitive mutants and hormone-insensitive mutants have also demonstrated a complex interplay of factors regulating the common signaling capabilities of sugar/hormone interactions. Characterization of sugar-signaling mutants in *Arabidopsis* has unraveled a complex signaling network that links sugar responses to two plant stress hormones, abscisic acid and ethylene, in opposite ways. Similar cross talk between sugar and other plant hormones in their signaling capabilities has been discussed.

Keywords

Abscisic acid • G proteins • Hexokinase • Hormones • Signaling • Sugars • Trehalose

5.1 Introduction

Sugars, the molecules of fundamental importance for life, act as the primary carriers of captured energy from the sun. They have been found to modulate a range of vital processes of plant

W. Shahri • S.S. Ahmad • I. Tahir (✉)
Plant Physiology and Biochemistry Research Laboratory, Department of Botany, University of Kashmir, Srinagar 190006, Jammu and Kashmir, India
e-mail: waseem.bot@gmail.com; syedsabhiindrabi@gmail.com; inayatullahtahir@gmail.com

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growth and development including seed germination, seedling development, root/leaf differentiation, floral transition, fruit ripening, embryogenesis, and senescence, as well as regulation of responses to light, stress, and pathogens. During germination and early seedling development, sugars are known to repress nutrient mobilization, hypocotyl elongation, cotyledon greening/expansion, and shoot development (reviewed in Leon and Sheen 2003) and that sugar starvation in plants activates lipid mobilization, fatty acid transfer, and peroxisomal β-oxidation (Hooks et al. 1995). Using transcriptome profiling analysis in *Arabidopsis*, it has been shown that sucrose (a sugar source) plays an important role in the activation of oxidative-stress genes, such as catalase (Contento et al. 2004). As it is a well-known fact that plant growth and development is under the tight regulation of the environmental conditions that in turn influence the availability of photosynthetic carbon in the form of carbohydrates. These developmental processes are however required to meet the carbon or energy demands of the system, and as such, the production, utilization, mobilization, and allocation of these photosynthates (carbohydrates) in various tissues at different stages of development are therefore highly regulated.

Sugar production in plants mainly involves photosynthetic conversion of light energy into chemical bond energy of organic molecules, utilizing the conventional photosynthetic pathway, and as such, the process of photosynthesis carries the vital importance to plants. The sugar status of a plant has been found to coordinate internal regulators and external environmental cues that in turn govern vital processes of growth and development (Koch 1996; Sheen et al. 1999; Smeekens 2000). Moreover, sugar metabolism is a dynamic process as sugar concentrations have been found to alter dramatically during development and in response to environmental signals, diurnal changes, and biotic/abiotic stress (Rolland et al. 2006). Sugars serve both as an energy source and as signaling components, e.g., sucrose serves as a main transport carbohydrate in plants and also as a signal molecule that can regulate gene expression and plant development (Baier et al. 2004). Similarly in *Vicia faba* embryos, gradients of sugars have been reported to correlate spatially with mitotic activity (Borisjuk et al. 1998), and in *Arabidopsis*, D-type cyclin gene expression has also been found to be regulated by sugars (Riou-Khamlichi et al. 2000) which points out that sugars provide positional information to the cell cycle machinery and different developmental programs. As far as the effect of sugars on floral transition is concerned, studies have revealed that increased leaf carbohydrate export and starch mobilization are required for flowering (Corbesier et al. 1998) and that the addition of sugar source (sucrose at optimum concentrations) can rescue the late-flowering phenotype of several mutants and even promotes leaf morphogenesis and flowering in the dark (Araki and Komeda 1993; Zhou et al. 1998; Roldan et al. 1999). Ohto et al. (2001) has reported that sugars may control floral transition by positively and negatively regulating the expression of floral identity genes. Thus, sugars, in addition to their essential roles as substrates of carbon and energy metabolism, have important hormone-like functions or as primary messengers in plant signal transduction. Sugars have been reported to affect the expression of many genes involved in photosynthesis, glycolysis, nitrogen metabolism, sucrose and starch metabolism, defense mechanisms, and cell cycle regulation, and therefore, studies have been and are being made to reveal the sugar-sensing and signal transduction pathways (Rolland et al. 2006; Bolouri-Moghaddam and Van den Ende 2012). In the present review, we attempt to consolidate the information regarding the role of sugars as signaling molecules in plants and to discuss the different dimensions of the sugar signal transduction pathway.

5.2 Sugars as Signaling Molecules

Sugars such as glucose, fructose, or sucrose have been recognized as important signaling molecules in plants, in addition to their typical roles as carbon and energy sources (Koch 2004; Rolland et al. 2006; Bolouri-Moghaddam et al. 2010). Of the various sugars, sucrose is the most important

Fig. 5.1 Role of sugars in plant growth and development

metabolite in resource allocation system of plants and is the predominant form of carbon transported to the heterotrophic tissues (Chiou and bush 1998). Although it has been reported to regulate plant growth and development, differential gene expression, and stress-related responses, at the same time it is also emerging as a candidate signaling molecule in plant innate immunity (Gómez-Ariza et al. 2007; Birch et al. 2009; Wind et al. 2010). In many cases, the effects of sucrose have been shown to be completely mimicked by hexoses (glucose and fructose) as is reported in case of photosynthesis genes where hexoses at lower concentrations have been found to mimic their sucrose repression (Sheen et al. 1999). It is also indicative of the fact that in such a case sucrose is not the direct signaling molecule instead its hydrolysis products (glucose or fructose) might have the signaling capabilities. It has been reported that some plant families even use raffinose-family oligosaccharides and small fructans as their main transport sugars (Keller and Pharr 1996; Wang and Nobel 1998; Zuther et al. 2004). There has also been a growing recognition that free glycans (oligogalacturonides, chitosan, or chitin oligosaccharides) are also used as signals for the initiation of a wide variety of biological processes involving growth, development, and defense responses of plants and animals (Etzler and Esko 2009) (Fig. 5.1).

5.2.1 Hexose Signaling

The pivotal role of sugars as signaling molecules is illustrated by the variety of sugar-sensing and signaling mechanisms discovered in microorganisms (bacteria and yeast) and plants (*Arabidopsis*) (Rolland et al. 2001; Moore et al. 2003; Yanagisawa et al. 2003). Sugar signals have been reported to be generated either by carbohydrate concentration or by relative ratios to other

metabolites, such as C:N or by flux through sugar-specific sensors and/or transporters (Coruzzi and Bush 2001; Palenchar et al. 2004; Buttner 2010). To activate signal transduction pathways, a signaling molecule first has to be sensed. Nevertheless, dual function of sugars (as a nutrient and a signaling molecule) complicates the analyses of the mechanisms involved and the elucidation of the initial sugar sensor involved in signaling. However, the involvement of transporters like glucose sensors (Snf3 and Rgt2), a G protein-coupled receptor (Gpr1), and more importantly hexokinase (HXK) function has been reported (Rolland et al. 2001, 2002). Multiple sugar sensors/receptors are known to exist, with hexokinase (HXK) being the first to be documented in plants (Jang and Sheen 1997). Hexokinase is a multifunctional protein being both an enzyme, which catalyzes the first step of glycolysis (conversion of glucose to glucose-6-phosphate), and a glucose sensor. The sensing function of the protein has been found to be dependent on the enzymatic function in a similar manner as its yeast counterpart. The difference, however, being that overexpression of the yeast hexokinase in plants only enhances its catalytic activity (Rolland et al. 2006) and not the signaling capability. Hexose phosphorylation by hexokinases has been considered to be an essential step in sugar metabolism, involving at least two classes of glucose- and fructose-phosphorylating enzymes (hexokinases and fructokinases). Using transgenic and mutational approach, the functions of several *HXK* and *FRK* genes have been investigated wherein the sensing roles of HXK and developmental roles of FRK have been documented (Granot et al. 2013). Much progress has been made to reveal the molecular mechanisms underlying sugar sensing and signaling in plants, particularly the demonstration of hexokinase (HXK) as a glucose sensor (Sheen et al. 1999; Smeekens 2000). The isolation and characterization of the *Arabidopsis gin2* mutants have clearly identified hexokinase (AtHXK1) as a core component in plant sugar sensing and signaling. Interestingly, these mutants were found to have partial glucose kinase activity. Uncoupling of metabolic and signaling activity has been confirmed by the analysis of two catalytically inactive *AtHXK1* alleles (one deficient in ATP binding and the other deficient in phosphoryl transfer). Both these alleles have been reported to sustain wild-type growth, repression of photosynthetic gene expression, and hormone (auxin and cytokinin) responsiveness when expressed in a *gin2* background (Moore et al. 2003). These findings suggested the existence of other hexokinases (AtHXK2) and hexokinase-like proteins (AtHXL). In the *Arabidopsis* genome, six *HXK* and *HXK* like (*HXKL*) genes have been identified. One of the hexokinase-like proteins has been found to have detectable kinase activity and was therefore named as AtHXK3. More complex functions of HXK are being anticipated in rice, in which ten functional *HXK* homologs have been identified. The role of hexokinase as a glucose sensor has also been revealed by the use of various glucose analogs (2-deoxyglucose, mannose, 6-deoxyglucose, and 3-O-methyl glucose). 2-Deoxyglucose and mannose act as substrates for hexokinase and were found to mimic glucose signaling in the regulation of photosynthetic and glyoxylate genes, while non-metabolizable 6-deoxyglucose and 3-O-methylglucose were found to be effective in mimicking the signaling response in the regulation of invertase and patatin genes. These findings support the existence of both hexokinase and non-hexokinase sugar sensors (Sheen et al. 1999). The identification of two hexose transporter-like sensors, SNF3 and RGT2, that mediate glucose regulation of glucose transporter genes in yeast has suggested that similar hexose sensors might exist in plants (Johnston 1999; Lalonde et al. 1999). In *Arabidopsis*, three glucose transporter-like proteins have been identified as potential candidates and it has been suggested that distinct hexose sensors might be used for diverse hexose signaling pathways in plants. Hohmann et al. (1999) proposed that hexokinase undergoes a conformational change after binding to its substrate glucose or other hexoses, and this regulation might be an essential mechanism of the sensing process. This conformational change of HXK might resemble the analogous ligand-induced conformational change of a typical receptor that allows modification of protein–protein interactions to trigger a signaling

cascade. It has been also postulated that altered ATP/ADP ratios or altered cytosolic phosphate ion concentration, as a result of hexokinase activity, might have a signaling function, but the experimental verifications are still pending (Sadka et al. 1994).

HXK and HKL protein localization has been suggested to play an important role in their functions as has been evidenced from the association of different hexokinase or hexokinase-like proteins with different cell organelles (particularly chloroplast and mitochondria; Borchert et al. 1993; Galina et al. 1995; Wiese et al. 1999), whether it being the HXK protein association with mitochondria in *Arabidopsis* and maize or an inner-plastidic HXK in tobacco (Galina et al. 1995, 1999). Moreover, AtHXK1 has been reported to translocate to nucleus as well. Here it is important that the involvement of cytosolic hexokinases as hexose sensors has not been observed in the cytoplasm, and it has been proposed that hexoses are sensed only when produced in the endomembrane system (Golgi–endoplasmic reticulum). The apoplastic and vacuolar targeted invertases are thought to play an important role as they are enzymatically active in these compartments and result in the generation of monosaccharides which are then sensed. Hexoses generated in either the endomembrane system or in plastids are then transported into the cytosol with concomitant phosphorylation by signaling hexokinases. Therefore, these transport-associated hexokinases are capable of signaling, while the hexoses produced in the cytosol are not (Halford et al. 1992; Koch 1996). Plant hexokinases have been grouped into two types: type A kinases (such as PpHXK1 and two *Arabidopsis* HXLs), which have a predicted chloroplast transit peptide, and type B kinases (such as AtHXK1 and AtHXK2), which have a membrane anchor. In addition to HXKs, plants are also reported to contain several fructokinases, some of which have been implicated in sugar sensing. Three fructokinase (*FRK*) genes and several *FRK*-like genes have been identified in *Arabidopsis*. In tomato, fructokinase transcripts, *FRK1* and *FRK2*, have been found to be induced by exogenous application of sugars (glucose, fructose, as well as sucrose; Kanayama et al. 1998). Although it is generally believed that FRKs play metabolic roles, the identification of an *frk2* null mutation in *mig* mutant suggests that FRK might be involved in sugar sensing (Pego and Smeekens 2000). This is also confirmed from the fact that glucose-insensitive (*gin*) mutants in Arabidopsis showed glucose insensitivity but were sensitive to fructose and sucrose. The role of fructose as signaling molecule has been demonstrated in *Arabidopsis* where it induces seedling developmental arrest and interacts with plant hormones (abscisic acid and ethylene) in a similar manner to that of glucose. Although earlier studies have suggested the role of fructokinase (an enzyme which phosphorylates fructose in the same manner as hexokinase phosphorylates glucose) in fructose signaling (Pego and Smeekens 2000; Odanaka et al. 2002; German et al. 2003), recent studies have demonstrated the role of fructose insensitive1 (FINS1) or fructose-1,6-bisphosphatase (F6BP) as a putative signaling component. It has been reported that role of FINS1 as a signaling component is independent of its catalytic activity and that fructose signaling is independent of HXK function (Cho and Yoo 2011).

Hexokinase 1 (HXK1) has been implicated to be an evolutionarily conserved glucose sensor that integrates nutrient and hormone signals to govern gene expression and plant growth in response to environmental cues (Cho et al. 2006). Based on the role of hexokinases, three distinct glucose signal transduction pathways have been identified in plants (Xiao et al. 2000).

1. Hexokinase dependent and metabolism independent
2. Hexokinase dependent and metabolism dependent
3. Hexokinase independent

The regulation of photosynthetic genes by hexokinase 1 (HXK 1) provides an excellent example of hexokinase-dependent (metabolism-independent) pathway. Inside the nucleus, HXK1 has been found to interact with the vacuolar H^+-ATPase B1 (VHA-B1) and the 19S regulatory particle of proteasome subunit (RPT5B) in a glucose-dependent manner to form an HXK1-nuclear complex that directly binds to promoters

of glucose-regulated genes (Cho et al. 2006). The second pathway is glycolysis dependent and has been found to be sustained by the heterologous yeast HXK2 activity, e.g., the glucose induction of *PR1* and *PR5* gene expression or the sugar-induced expression of senescence-associated gene *SAG21* (Noh and Amasino 1999; Xiao et al. 2000). The third pathway, i.e., hexokinase-independent pathway, is represented by glucose induction of *CHS*, *PAL1*, and genes encoding AGPase as well as by glucose repression of aspartic synthase (*ASN1*). Ryu et al. (2004) also reported that the induction of carotenoid biosynthesis genes by glucose also involves hexokinase-independent pathway. This pathway has been reported to involve (cyclic AMP-protein kinase) cAMP–PKA signaling. cAMP synthesis by adenylate cyclase via glucose activation involves a dual function. Evidences have suggested that multiple hexose kinases (hexokinases, HXK1 and HXK2, or glucokinase, GLK1) on one hand play a regulatory role through activation of small Ras G proteins (required for adenylate cyclase activity), while on the other hand, these extracellular sugars (glucose or sucrose) are sensed by the G protein-coupled receptor (GPCR) system consisting of GPR1 receptor, GPA2 (a heterotrimeric Gα-protein) and RGS1/RGS2, negative regulators of G protein signaling (Chen et al. 2003; Chen and Jones 2004; Lemaire et al. 2004). Mutants in G protein-interacting membrane protein (*RGS1*) gene have been generated and found to have impaired glucose sensing and that the mutants in G protein α-subunit gene GPA1 were found to have impaired glucose sensitivity (Huang et al. 2006; Grigston et al. 2008). Like *Saccharomyces cerevisiae*, G protein signaling elements have also been identified in *Arabidopsis*, which contained a G_α subunit (AtGPA1), a G_β subunit (AGB1), one or two G_γ subunits, and a regulator of G-signaling protein (AtRGS1). The *Arabidopsis* heterotrimeric GPA1 complex (consisting of G_α, G_β, and G_γ subunits) has been implicated to play an important role in abscisic acid signaling, in biotic/abiotic stress, in germination and early development, as well as in glucose signaling (Tuteja and Sopory 2008). Moreover, AtRGS1 has been reported to comprise a *C*-terminal RGS domain coupled to an *N*-terminal domain with a predicted seven-transmembrane topology which interacts with the AtGPA1 at the plasma membrane and functions as a GTPase activating protein (GAP) for AtGPA1 (Ritchie et al. 2002; Choi et al. 2005; Finkler et al. 2007). Recently, Grigston et al. (2008) have demonstrated that AtRGS1, a putative extracellular receptor for D-glucose aided with the heteromeric G protein complex, mediates the steady-state level of transcripts of some sugar-related genes in a G protein-coupled signaling network in *Arabidopsis*.

5.2.2 Sucrose Signaling

In addition to hexoses (glucose and fructose), other sugars like sucrose, trehalose, some rare sugars (psicose and D-allose), and the sugar-analogs 2,5-dideoxy-2,5-imino-D-mannitol (DMDP, a fructose analog) are reported to have signaling capabilities (Lalonde et al. 1999; Smeekens 2000). These have been found to stimulate the plant immune system and upregulate various genes involved in plant growth and development. Moreover, it has been suggested that these compounds act as signals through hexokinase-dependent/independent pathways (Birch et al. 1993; Vaughn et al. 2002; Derridj et al. 2009; Kano et al. 2011). Compared to hexose signaling, sucrose signaling is far more complex. Sucrose can readily be hydrolyzed to the corresponding hexoses (fructose and glucose), and as such, it becomes difficult to separate the signaling function of sucrose from its hydrolysis products, and thus, the nature of sucrose signaling can often be attributed to the hexose-dependent pathways. However, the identification of some sucrose-specific genes (whose expression is specifically regulated by sucrose and not by hexoses) points to an HXK-independent sucrose-specific signaling pathway, e.g., sucrose-specific expression of a gene encoding a proton–sucrose symporter in sugar beet (Vaughn et al. 2002), the sucrose-mediated translational inhibition of the ATB2/bZIP11 transcription factor in *Arabidopsis* (Wiese et al. 2004; Rosa et al. 2009),

and the posttranscriptional control of a leucine zipper transcription factor (Rook et al. 1998). In addition, studies on starch synthesis in potato tubers and on seed development in transgenic *Vicia narbonensis* also support the involvement of sucrose-specific signals in the differentiation and synthesis of storage products (Geiger et al. 1998; Weber et al. 1998). Nevertheless, Loreti et al. (2001) have reported that the expression of α-amylase gene in barley embryos is modulated by both glucose and sucrose independently. Therefore, sucrose can be sensed as a signal directly or, alternatively, a signal can arise via its hexose cleavage products, i.e., glucose or fructose (Chiou and Bush 1998; Li et al. 2011; Eveland and Jackson 2012). It is also obvious that sucrose could have a signaling mission distinct from that of hexoses and therefore could be perceived by different types of sensors/receptors. The nature of sucrose sensor involved in sucrose signaling is still obscure; however, sucrose transporter SUT2/SUC3 has been proposed to act as a sensor in analogy to SNF3 and RGT2 glucose sensors in yeast (Eckardt 2003). Two sucrose transporter cDNAs (*CitSUT1* and *CitSUT2*) have been isolated and characterized from citrus. They have been reported to encode putative proteins (CitSUT1 and CitSUT2) of 528 and 607 amino acids, respectively, and that both proteins contain 12 predicted transmembrane domains. The expression of *CitSUT1* in mature leaf discs has been found to be repressed by exogenous sucrose, glucose, mannose, and the glucose analog 2-deoxyglucose, but not by another glucose analog 3-O-methylglucose, indicating a hexokinase (HXK)-mediated signaling pathway, while *CitSUT2* expression has been found to be unaffected by exogenous importing sugars, suggesting different physiological role for this sucrose transporter. The expression patterns of citrus sucrose transporters also showed temporal regulation as *CitSUT2* has been found to be expressed in young leaves and *CitSUC1* in the mature leaves (Li et al. 2003). Remarkably, the S1 group bZIP transcription factors (bZIP1, bZIP2, bZIP11, bZIP44, and bZIP53) have been found to be translationally repressed by sucrose, and this repression has been reported to be dependent on an upstream open reading frame (uORF) present in the 5′ leader of the bZIP mRNAs (Rahmani et al. 2009; Weltmeier et al. 2009) that encodes a highly evolutionary conserved sucrose control peptide (SC peptide). The translational repression of bZIP transcription factors by sucrose has recently been reported to depend on conditional stalling of a ribosome translating the SC peptide wherein the stalled ribosomes on the mRNA have been found to inhibit translational initiation of the bZIP encoding ORF. In general, hexoses are considered to have greater signaling potential in promoting organ growth and cell proliferation, while sucrose has been suggested to be typically associated with differentiation and maturation. It has also been postulated that relative ratios of hexoses to sucrose are perceived and maintained by sucrose metabolic enzymes, for which different isoforms act in a spatio-temporal manner to control and coordinate the fine-tuning of growth during different phases of development (Xu et al. 1996; Borisjuk et al. 2002; Koch 2004).

5.2.3 Trehalose Signaling

Trehalose is another important sugar with signaling capabilities. It normally acts as an osmoprotectant which counters the effects of dessication from drought, salt, or low-temperature stress (Crowe et al. 1992). In *Arabidopsis*, T6P accumulation has been found to be associated with increased anthocyanin accumulation during later stages of leaf development (Wingler et al. 2012). Since anthocyanins accumulate under high carbon supply, it has been suggested that T6P signals high sugar availability and thereby stimulating the anthocyanin biosynthetic pathway. Moreover, plants overexpressing microbial trehalose biosynthetic genes have been reported to have altered carbohydrate metabolism and morphological defects like stunted growth (Romero et al. 1997; Garg et al. 2002; Schluepmann et al. 2003, 2004). These phenotypes are thought to result from changes in carbon allocation between sink and source tissues and it has been speculated that trehalose might be

involved in sugar signaling (Paul et al. 2008). In plants, trehalose biosynthesis occurs in two steps. In the first step, trehalose-6-phosphate (T6P; an intermediate) is formed from UDP-glucose and glucose-6-phosphate by trehalose-6-phosphate synthase (TPS). The second step involves the conversion of T6P to trehalose by trehalose-6-phosphate phosphatase (TPP) (Cabib and Leloir 1958). About 11 trehalose phosphate synthase (*AtTPS1-11*) genes and 10 trehalose-6-phosphate phosphatase (*AtTPPA-J*) genes have been identified in *Arabidopsis* (Leyman et al. 2001). AtTPS proteins have been reported to carry both trehalose-6-phosphate synthase (TPS)- and trehalose-6-phosphate phosphatase (TPP)-like domains. Among the Arabidopsis TPSs, only AtTPS1 is reported to have demonstrable TPS activity, while other TPSs lack both TPS and TPP activities (Ramon et al. 2009). Similarly in rice, two active isoforms of TPS1 (*OsTPS1a* and *OsTPS1b*) have been identified (Zang et al. 2011). Based on the homology of Arabidopsis TPSs with the yeast trehalose-6-phosphate synthases (ScTPS), they have been classified into two distinct subfamilies (Ponnu et al. 2011).

Class I Subfamily: It involves AtTPS1–AtTPS4 which are characterized by highest overall symmetry to ScTPS1, and in them, the TPP-like domain is only weakly conserved. AtTPS1 differs from the other class I TPSs in that it contains an auto-inhibitory *N*-terminal extension that restricts its activity in vivo.

Class II Subfamily: It involves AtTPS5 to AtTPS11 which displays more similarity to ScTPS2. These are reported to contain conserved TPP motifs. On the other hand, all trehalose-6-phosphate phosphatases (TPPs) lack the *N*-terminal TPS-like domain and contain only the conserved TPP domain with significant similarity to the highly conserved phosphatase box in the *C*-terminal part of ScTPS2 (Lunn 2007). Out of the ten TPPs in *Arabidopsis*, only *AtTPPA* and *AtTPPB* genes have been shown to encode active TPP enzymes (Vogel et al. 1998). Similarly, rice *TPP2a* and maize *RAMOSA3* (Habibur Rahman Pramanik and Imai 2005; Satoh-Nagasawa et al. 2006; Shima et al. 2007) have also been shown to encode active TPPs.

Evidences accumulated so far support the fact that T6P, rather than trehalose itself, has signaling capabilities. Developmental processes that are regulated by T6P range from embryo development to leaf senescence. Some of these processes have been found to be regulated in interaction with phytohormones, such as auxin (ÓHara et al. 2013). T6P has been found to regulate starch synthesis via redox activation of ADP-glucose phosphorylase (which catalyzes the first step in starch biosynthesis) and has recently been shown to inhibit the KIN10/11 regulatory kinase (Lunn et al. 2006; Zhang et al. 2009; Wingler et al. 2012). The direct regulation of KIN10/11 by T6P has not been demonstrated, and hence, unknown additional signaling steps are thought to be involved. KIN10/11 proteins have been found to regulate gene expression through specific transcription factors of which a small group of bZIP G-box binding transcription factors are of particular importance. The transcription factors have been reported to bind to the promoters of genes regulated by the KIN10/11 signaling pathway. More importantly, bZIP proteins have been found to harbor conserved motifs for phosphorylation by AMPK/SNF1-like kinases which make them vulnerable to direct phosphorylation that might regulate the activity of these transcription factors (Baena-Gonzalez et al. 2007; Hanson et al. 2008). However, more experimental details are required to substantiate the conclusion.

T6P, as a signaling molecule, allows yeast hexokinase to perceive carbon status (Paul et al. 2001) where, as in Arabidopsis, no direct link between hexokinase inhibition and T6P has been observed (Eastmond et al. 2002). It has been suggested that a protein kinase, sucrose non-fermenting-related kinase-1 (SnRK1), might serve as a link between the two (Schluepmann et al. 2004). The role of SnRK1 in regulating plant metabolism has been well established. It has been demonstrated to play an important role in starch breakdown as the expression of α-amylase in wheat and rice embryos during sugar starvation requires the SnRK1 activity. However, overexpression of SnRK1 in potato tubers has been reported to increase the expression of sucrose synthase and

AGPase genes (resulting in increased starch content) which demonstrates that SnRK1 is also involved in activating starch synthesis (Laurie et al. 2003; McKibbin et al. 2006; Lu et al. 2007). It has been reported to bring direct phosphorylation leading to inactivation of various metabolic enzymes including 3-hydroxymethylglutaryl-CoA reductase, sucrose phosphate synthase, nitrate reductase, and trehalose-6-phosphate synthase (Polge and Thomas 2007; Halford and Hey 2009). Zhang et al. (2009) have reported that T6P inhibits the catalytic activity of SnRK1 in vitro at physiological concentrations in *Arabidopsis* seedlings, but not in mature leaves. Such a variation in the regulation of SnRK1 by T6P has also been reported during wheat grain development (Martínez-Barajas et al. 2011). It has been speculated that a protein factor, present only in growing tissues like seedlings and young leaves of *Arabidopsis* and in cauliflower florets, underlies these developmental changes in the inhibition of SnRK1 by T6P. The regulation of SnRK1 itself in response to the availability of metabolites has not been fully demonstrated. Jossier et al. (2009) point out that phosphorylation of SnRK1 in response to glucose leads to its activation. Here the role of evolutionary conserved 14-3-3 proteins has been documented. The 14-3-3 proteins have been reported to bind specifically to phosphorylated substrates and thus controlling the enzyme activities, subcellular location, and protein–protein interactions required for such signal transduction pathways (Finnie et al. 1999; Sehnke et al. 2002). It has also been proposed that the loss of 14-3-3 protection and the resulting proteolysis bring about the major metabolic shift to reduce nitrate assimilation and sugar synthesis upon sugar starvation (Cotelle et al. 2000). Moorhead et al. (1999) also reported the interaction of plant trehalose-6-phosphate synthase with 14-3-3 proteins, thereby supporting a role for trehalose-6-phosphate in the starvation response, while Paul et al. (2001) suggested that loss of 14-3-3 binding releases trehalose-6-phosphate from the trehalose synthesis complex under conditions of low carbon supply. Recently, the transcription factor bZIP11 has been identified as an important component of the T6P/SnRK1 regulatory pathway (ÓHara et al. 2013). In *Arabidopsis* seedlings, an interaction between bZIP11 and SnRK1 has been found in response to trehalose feeding, and a subtle mechanism including a regulatory loop that regulates growth in response to sucrose through an increase in T6P and inhibition of SnRK1 and therefore of bZIP11-dependent gene expression has been proposed. It has been suggested that too much T6P in the absence of a sufficient sucrose supply can result in carbon deficit because of over-activation of biosynthetic pathways and reduced carbon salvage through catabolic pathways, whereas too little T6P inhibits growth because of the downregulation of biosynthetic pathways required for growth (Delatte et al. 2011).

5.2.4 Glycan Signaling

Apart from sugars like glucose, fructose, sucrose, or trehalose, glycans or oligosaccharins have been reported to act as signaling molecules in plants. The first indication of glycans or oligosaccharins as signaling molecules came from the studies on plant defense responses where it has been shown that plant or pathogen cell wall-released glycans elicit the defense response. It has been reported that specific free glycans in picomolar to micromolar concentrations have signaling capabilities for the initiation of a number of biological processes, particularly in the defense response of plants and the initiation of the nitrogen-fixing *Rhizobium*–legume symbiosis. Glycan signaling systems have been suggested to involve various glycoconjugates as has been demonstrated by the changes in cytoskeleton, gene transcription, and enzyme activation on the addition of O-GlcNAc to cytoplasmic and nuclear proteins. Specific receptors on the plasma membrane have been found to recognize the glycans. Experimental studies with plant cell cultures and isolated plasma membranes have demonstrated the existence of specific cell-surface or membrane-binding sites with binding

specificities similar to those required for biological behavior. The recently identified protein CEBiP (a 75-kDa plasma membrane protein from cultured rice cells), as a candidate for such receptors, is a chitin oligosaccharide elicitor binding protein that binds chitin elicitors. This protein has been recently purified and the corresponding gene cloned. The protein was found to be a membrane protein without any appreciable portion on the cytoplasmic side of the membrane, suggesting that it might be part of an elicitor–receptor complex. Further studies have shown that reduced expression of the corresponding gene resulted in suppression of the defense response. Similarly, the role of glycosaminoglycans (GAGs) as signaling molecules is also evident from the fact that they interact with receptor tyrosine kinases and/or their ligands and facilitate changes in cell behavior, e.g., hyaluronan oligosaccharides have been found to bind to specific membrane proteins (such as CD44) resulting in clustering of CD44 and activation of several kinases (such as c-Src and focal adhesion kinase [FAK]) to bring out phosphorylation resulting in the alteration in the interaction of the cytoplasmic tail of CD44 with regulatory and adaptor molecules that modulate cytoskeletal assembly/disassembly and cell survival and proliferation. Such a signaling by hyaluronan oligosaccharides has been reported to depend on the degree of polymerization of the glycans, with low-molecular-weight chains more active than high-molecular-weight chains. Likewise, glycosphingolipids are also known to form lipid rafts, which act as a platform for sequestering signaling receptors, or can associate with receptor tyrosine kinases to modulate their activity (Etzler and Esko 2009).

5.3 Sugar Signaling and Gene Regulation

Sugars are known to activate various pattern recognition genes (Johnson and Ryan 1990; Herbers et al. 1996a, b; Rolland et al. 2002). A wide variety of genes have been found to be regulated by sugars at the transcriptional level, e.g., genes involved in photosynthesis, carbon/nitrogen metabolism, stress responses, and secondary metabolism. The initial candidate-gene approaches have demonstrated that variations in sugar levels could significantly modify the expression of genes related to abiotic stress. For example, the expression of chalcone synthase, which allows the synthesis of photoprotective anthocyanins and superoxide dismutase, has been reported to be induced by glucose (Koch 1996). Similarly, sucrose synthase gene (*Sus1*) encoding sucrose synthase protein (SuSy) has been found to be regulated by glucose and mannose. Based on the effects of hexokinase inhibitor, *N*-acetyl glucosamine, and using the mutational approach (plants with impaired *HXK1* expression), it has been shown that both the sugars (glucose and maltose) employ different regulatory mechanisms, suggesting that the regulation of *Sus1* involves multiple transduction pathways. At low concentration of sugars, hexokinase is thought to be involved, while at higher concentrations, the involvement of osmoticum pathway has been suggested (Ciereszko and Kleczkowski 2002). Moreover, the role of phosphoprotein in mediating the signal transduction has also been confirmed but the signaling pathway it affects has not been clearly demonstrated for *Sus1* regulation (Ciereszko et al. 2001). It has been further reported that the effect of sucrose on *Sus1* gene is not a direct effect of sucrose; instead, it is due to the effect of glucose (a cleavage product of sucrose) through hexokinase-dependent pathway. Using mutant analysis approach in *Arabidopsis*, a positive correlation between glucose feeding and aliphatic glucosinolate biosynthesis has been reported. Aliphatic glucosinolates have been demonstrated to play an important role in plant–herbivore interactions and nonhost resistance in the *Arabidopsis–Pseudomonas* pathosystem. Glucosinolate biosynthesis genes *CYP79F1* and *CYP79F2* have been found to be upregulated by glucose. However, the upregulated expression of these genes in double mutant *myb28/myb29* has not been reported, suggesting that the glucose-mediated aliphatic glucosinolate biosynthesis genes are regulated via MYB28/MYB29 transcription factors. Moreover, the total aliphatic glucosinolate content and the expression level of *MYB28* and *MYB29* have been found to be

substantially reduced in the glucose-insensitive (*gin2-1)* mutant, the ABA-insensitive 5 (*abi5-7*) mutant, and sugar-insensitive RGS1 (regulator of G protein signaling 1) mutant (*rgs1-2)* which suggests the hexokinase and/or G protein involvement in mediating glucose signaling for glucosinolate biosynthesis. The evidence for glucose-specific induction of glucosinolate biosynthesis/accumulation is also evident from the fact that fructose or mannose has been found to be ineffective in mimicking the induction of glucosinolate genes (Miao et al. 2013). In cyanobacterium *Synechocystis*, Ryu et al. (2004) have shown that glucose induces expression of carotenoid synthesis genes in the dark. In this way, it mimics the effects of high light on carotenoid synthesis genes and thereby opens the possibility that glucose plays a role in the regulation of carotenoid synthesis in response to high light. The reports by Price et al. (2004) in *Arabidopsis* seedlings have also confirmed the glucose induction of several stress response genes including oxidative-stress-related genes, such as chalcone synthase, glucose-6-phosphate dehydrogenase, glutathione-S-transferases, and glutathione conjugate transporters. Similarly, a fine example of sugar regulation of genes at translational level is the sucrose repression of basic leucine zipper gene (*ATB2*) in *Arabidopsis*. For this repression, glucose and fructose individually or together have been found to be ineffective. The *ATB2* mRNA has been found to carry a complex leader containing small open reading frames and its deletion from the transcript has been reported to abolish the sucrose-mediated repression (Rook et al. 1998). These results indicate that a sucrose-specific signal controls translation repression of mRNA levels (Fig. 5.2).

A sugar-signaling cascade involves sugar sensors to feed information (sugar signaling) into signal transduction cascades to result in various types of plant responses. The signal transduction cascade has been found to involve various components like mitogen-activated protein kinases (MAPKs), calcium-dependent protein kinase (CDPK), protein phosphatases (PPs), Ca^{2+}, calmodulin, SnF1-related protein kinase (AtSR2), and transcription factors (Ishiguro and Nakamura 1994; Takeda et al. 1994; Ohto and Nakamura 1995; Ehness et al. 1997; Rook et al. 1998; Gupta and Kaur 2005). Sugar regulation of gene expression can be mediated at the transcriptional and post-transcriptional levels. Most progress has been made through the functional dissection of sugar-induced gene promoters. Whether it be the characterization of sucrose-responsive elements in the patatin class I promoter, SP8 motifs in the promoters of sweet potato sporamin and α-amylase genes, or sucrose-responsive sequences in some sucrose-inducible sucrose synthase genes (Liu et al. 1990; Ishiguro and Nakamura 1992, 1994; Grierson et al. 1994; Kim et al. 1994; Fu et al. 1995). Both positive and negative *cis*-elements have been found. Ishiguro and Nakamura (1994) have identified and cloned a gene *SPF1*, which encodes a DNA-binding protein that can recognize the SP8 motif in the sporamin and α-amylase gene promoters. It has also been reported that the gene encodes a negative regulator which is transcriptionally repressible by sucrose. *SPF1* homologs have been identified and isolated from cucumber and *Arabidopsis* that were found to encode a WRKY domain transcription factor. W-box and G-box elements have been identified as essential motifs for these transcription factors (Kim et al. 1997). Likewise, glucose repression of rice α-amylase gene promoters has revealed multiple *cis*-elements important for sugar-related gene expression. In the promoter of a rice α-amylase gene *αAmy3*, major sugar response sequence (SRS) has been found to be located between 186 and 82 base pairs upstream of transcriptional site. Three essential motifs, i.e., the GC-box, G-box, and TATCCA element, within the SRS have been identified (Lu et al. 1998). A more complicated interaction has been shown in the regulation of photosynthetic genes, particularly the interactions between sugar and oxidative cues. In the absence of abiotic stress, sugars such as glucose or sucrose have been shown to repress photosynthesis-related genes (e.g., *psbA* or D1 protein accumulation) in plants and in cyanobacterial cells. In the cyanobacterium *Synechocystis*, glucose feeding has been found to derepress the steady-state mRNA levels of PSII genes and induces the destabilization of *psbA* transcripts. However, the enhancement of psbA gene expression (in dark) has been reported

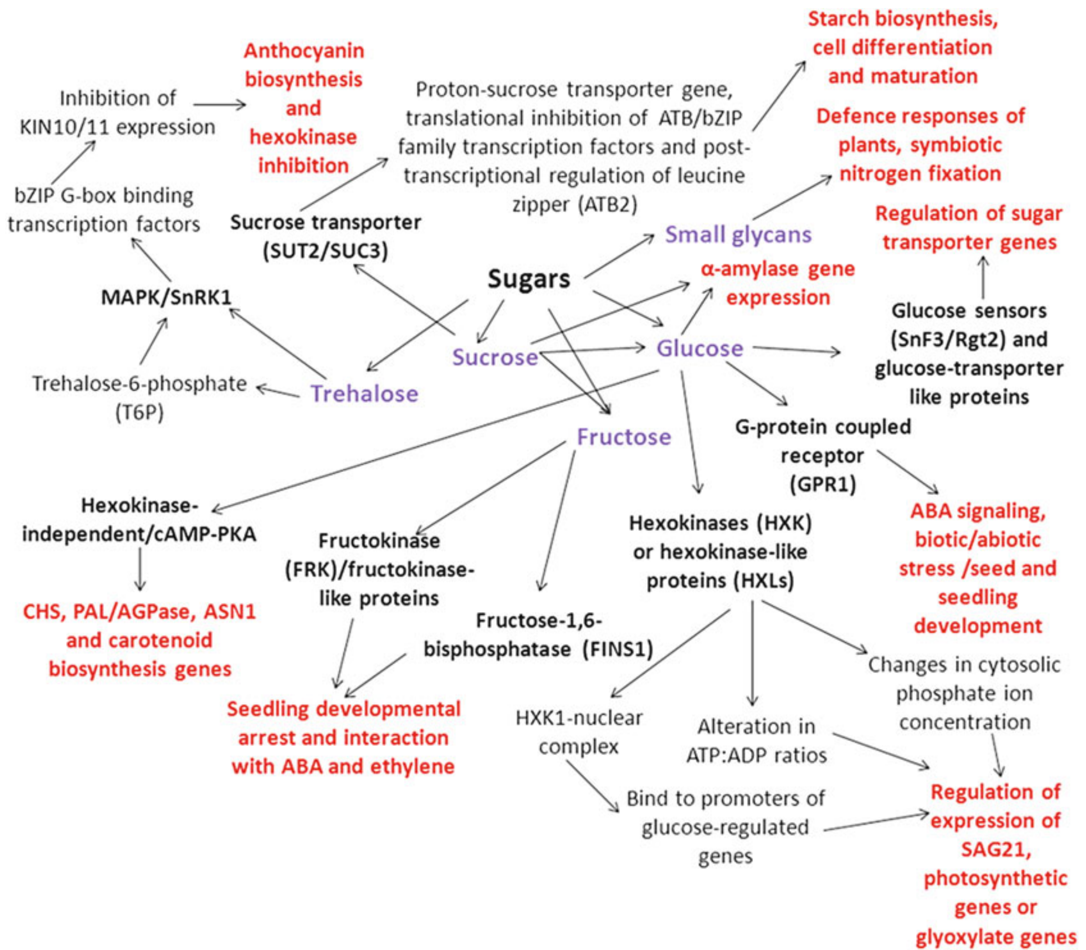

Fig. 5.2 Sugars: sensing and signaling capabilities

to occur in response to reactive oxygen species, hydrogen peroxide, or changes in the glutathione redox state (Couée et al. 2006). Another classical example of sugar-induced repression of photosynthesis-related genes is provided by the *Arabidopsis* seedlings where the increased accumulation of *psbA* mRNA and D1protein in the presence of atrazine has been reported (Sulmon et al. 2004). Atrazine treatment itself has negative effects on D1 protein levels; therefore, the derepression in the presence of sugar and atrazine might result from interactions between sugar and oxidative signaling cues. Thus, on the one hand, the effects of soluble sugars on gene expression are mediated through sugar-specific signaling pathways, and, on the other hand, these effects are linked to regulations by redox, ROS, light, stress, and photosynthesis electron transfer signals. These interactions have been found to be mediated through common target genes particularly photosynthesis genes (*psbA*), ROS defense genes (chalcone synthase, glutathione synthase, ascorbate synthesis gene, carotenoid synthesis gene), and stress defense gene (HSP). However, the interactions in their signal transduction pathways remain to be fully elucidated. Sugars have also been found to activate the genes encoding nitrate transporters, nitrate reductase, asparagine synthase (*ASN2*), and glutamine synthase (*GS*) which support the existence of relationship

between sugars and nitrogen metabolism (Sheen et al. 1999). However, a distinct asparagine synthase (*ASN1*) gene has been found to be repressed by sugars. Moreover, the glucose regulation of *ASN1* and *GS2* genes in transgenic *Arabidopsis* has been found to involve hexokinase-independent pathway. The importance of sugar and nitrogen balance in plant life has also been demonstrated in maize where high nitrate signals enhance the expression of photosynthesis genes for sugar production (Sakakibara et al. 1998; Sheen 1999). Similarly, in senescing *Arabidopsis* leaves, exogenously supplied sugars have been found to induce expression of the senescence-associated gene *SAG21* (in an HXK-dependent manner), while another well-characterized senescence marker, *SAG12*, has been found to be repressed by sugars (Noh and Amasino 1999; Xiao et al. 2000). It has been postulated that the regulation of different *SAGs* might be controlled differentially by other factors besides sugars, such as developmental state and hormones (He et al. 2001). Moreover, little is known about the actual transcriptional machinery underlying these responses and they have been suggested to involve diverse transcription factors (Sheen 1990, 1999).

5.3.1 Involvement of Protein Kinases

The involvement of protein kinases (PKs) and protein phosphatases (PPs) as important components in sugar signaling has been implied (Smeekens 1998). The discovery and development of specific protein kinase and phosphatase activators/inhibitors in the past decade have provided valuable tools to examine the involvement of protein phosphorylation/dephosphorylation in diverse signal transduction pathways (MacKintosh and MacKintosh 1994). It has been shown that PP1 and PP2A inhibitors mimic glucose repression of photosynthesis genes in maize leaf cells and in photoautotrophic cultures of *Chenopodium rubrum*, besides activating glucose/stress-inducible invertase and phenylalanine ammonia lyase genes (Ehness et al. 1997; Sheen 1999). Moreover, the transcriptional repression of large number of genes involved in metabolic processes (respiration, gluconeogenesis, or the alternative-carbon source metabolism and uptake) has been found to involve the "main glucose repression pathway" wherein the glucose sensor hexokinase (HXK2) has been reported to interact with Glc7-Reg1 protein phosphatse1 (PP1) complex to bring out dephosphorylation and inactivation of SnRK1 (Moreno et al. 2005). However, the differential effect of protein kinase inhibitor staurosporine on such signals suggests the involvement of different protein kinases in different transduction pathways. One such plant protein kinase is the SnF1-related protein kinase (SnRK1) with potential involvement in carbon metabolism and sugar signaling (Halford and Hardie 1998; Hardie et al. 1998). Four plant SnRKs from rye, tobacco (NPK5), and Arabidopsis (AtKIN10 and AtKIN11) have been identified and found to complement the glucose repression in yeast snf1 mutant (Sheen et al. 1999). The SnF1 protein kinase, an ortholog of mammalian AMP-activated protein kinase (AMPK), has been reported to be involved in derepression of gene expression under low glucose and starvation conditions probably through phosphorylation of Mig1 (a zinc-finger DNA-binding transcription factor). The phosphorylation of Mig1 results in the dissociation of Mig1 from the repressor complex and subsequent export to nucleus where it interacts with HXK2 to form a stable complex to recruit corepressor proteins (Moreno et al. 2005). In *Arabidopsis*, the activity of KIN10 and KIN11 has been shown to be of central importance in linking stress, sugar, and developmental signals to regulate metabolism, energy balance, growth, and survival under stress, which in turn are regulated by several factors, including cell wall-derived factors (Baena-Gonzalez et al. 2007; Li et al. 2007; Polge and Thomas 2007). Recently, a novel protein kinase, MsK4 (glycogen synthase kinase 3-like kinase), from *Medicago sativa* has been reported to be involved in stress signaling and carbon metabolism. It has been found to be a plastid-localized protein kinase, associated with starch granules, whose activity is induced by high-salt stress.

Moreover, plants overexpressing MsK4 have been found to accumulate more starch and carbohydrate content than those of wild-type plants, suggesting that MsK4 acts as an important regulator of carbohydrate metabolism to environmental stress (Kempa et al. 2007). The role of protein kinases in plant defense responses, induced by cell wall-derived oligogalacturonides, has also been documented (Ridley et al. 2001; Denoux et al. 2008). Also, some plant defense genes have been found to be upregulated in response to fungal invasion. Cell wall-derived oligogalacturonides, released from the plant cell walls, have been suggested to act as elicitors of the plant immune system and that the actual receptors for these signaling molecules probably involve wall-associated kinases (WAK1 and WAK2) that can transfer the signal across the plasma membrane (Brutus et al. 2010). Moreover, the link between WAKs and transcriptional/enzyme regulation has been found to be established by mitogen-activated protein kinases (MAPKs) like MPK3 and MPK6 (Kohorn et al. 2012).

5.3.2 Involvement of Calcium

The use of chemicals that inhibit calmodulin or Ca^{2+} ion channels has pointed out the involvement of Ca^{2+} ions in sugar signaling (Ohto et al. 1995). One such sugar-induced calcium-dependent protein kinase (associated with plasma membrane) has been identified in tobacco (Ohto and Nakamura 1995) which has been proposed to control the activity of sugar transporters located in the membrane. Calcium is also reported to be an essential component of the sucrose signaling pathway that leads to the induction of fructan synthesis (Martinez-Noel et al. 2006). Pharmacological studies with Ca^{2+} channel blockers (LaCl3), EGTA, and calmodulin inhibitors also provided additional evidence for the involvement of Ca^{2+} signaling particularly in the sugar induction of sporamin and α-amylase gene expression in sweet potato and of anthocyanin biosynthesis in cell suspension cultures of *Vitis vinifera* (Ohto and Nakamura 1995; Vitrac et al. 2000). In transgenic tobacco leaf discs expressing apoaequorin, sucrose has been found to induce increase in cytosolic levels of free Ca^{2+}. It has been suggested that increases in free cytosolic Ca^{2+} concentrations might be due to membrane depolarization caused by sugar–proton symport (Rolland et al. 2002). Further studies at cellular and molecular level are required to elucidate the precise role of Ca^{2+} in sugar signaling.

5.4 Sugar Signaling and Plant-Hormone Interactions

Recent genetic and molecular studies of sugar-signaling mutants in *Arabidopsis* have revealed multiple interactions between sugar and plant-hormone signaling which is evident from the fact that these mutants display phenotypes as are found in mutants deficient in hormone biosynthesis and signaling (ABA or ethylene), in addition to altered sugar responses; e.g., glucose-insensitive (*gin1*) mutant in *Arabidopsis* has been found to display phenotypes as displayed by constitutive triple response (*ctr1*) mutant. The ethylene receptor mutant (*etr1*) and ethylene-insensitive mutants (*ein2* and *ein3*) have been found to be hypersensitive to glucose, while constitutive triple response 1 (*ctr1*) mutant to be glucose insensitive. Also ethylene-insensitive mutant (*ein2-1*) of *Arabidopsis* has been reported to have increased anthocyanin accumulation in response to sucrose treatment, indicating a negative role of ethylene in the sucrose and fructose signaling pathways (Kwon et al. 2011). Moreover, the application of an ethylene precursor 1-aminocyclopropane-1-carboxylate (ACC) to wild-type plants, in the presence of excess exogenous glucose, has demonstrated that the glucose-dependent developmental arrest could be overcome by ethylene (Zhou et al. 1998) thereby paving ways for the growth-promoting role of ethylene in Arabidopsis. Interestingly, the transcription factor ethylene insensitive 3 (EIN3), a key regulator in ethylene signaling has also been shown to be differentially regulated by protein stability by glucose in a hexokinase (HXK1)-dependent manner (Yanagisawa et al. 2003) (Fig. 5.3).

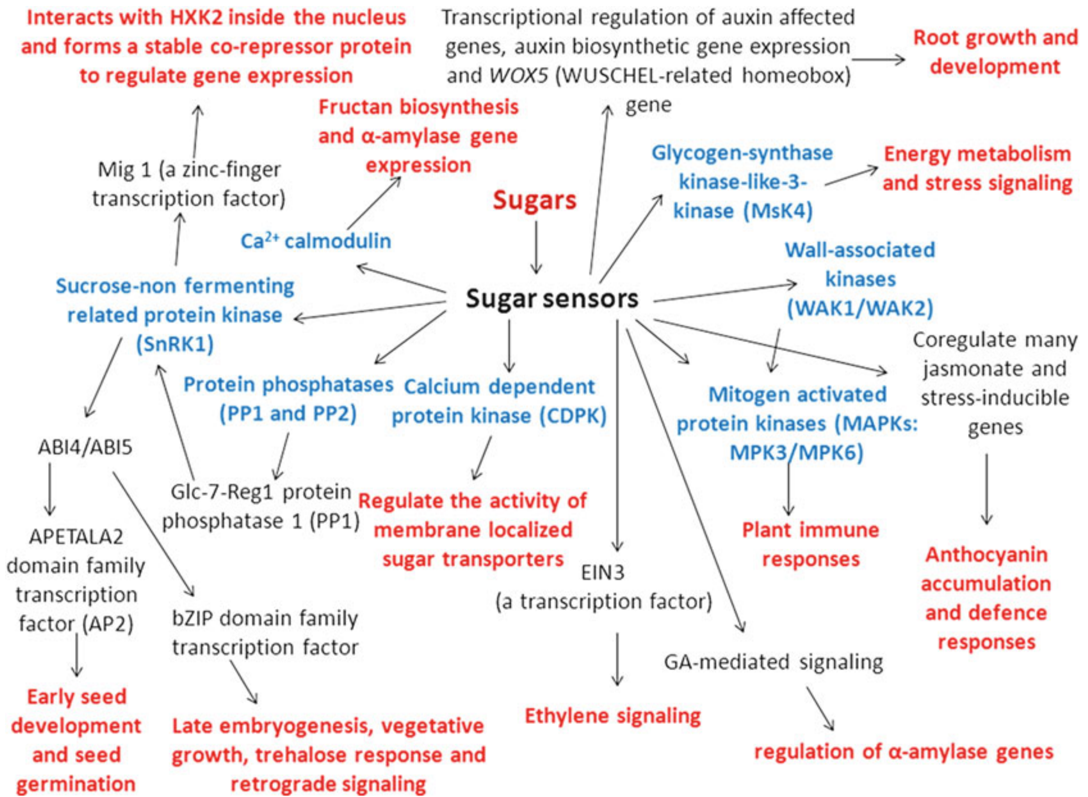

Fig. 5.3 Sugar signaling components and regulation of plant growth and development

Similar cross talk between sugar and abscisic acid (ABA) signaling has been demonstrated through the study of various glucose-insensitive mutants (*gin1* and *gin5*) in *Arabidopsis*. It has been found that the sugar-insensitive *gin1* and *gin5* mutants show reduced seed dormancy and wilty phenotypes as displayed by ABA-deficient (*aba*) mutants and that the ABA-deficient mutants (*aba1*, *aba2*, *aba3, abi4,* and *abi5*) also displayed a *gin* phenotype. Further studies on sugar-signaling mutants (*gin1*, *gin5*, *isi4,* and *sis4*) have also revealed that these mutants contain lower endogenous ABA levels than wild-type plants and that the addition of exogenous ABA (at physiological concentrations) reverts back the sugar sensitivity in these mutants (Leon and Sheen 2003). The characterization of the *gin6* mutant in *Arabidopsis* has resulted in the identification of *ABI4* as a candidate for the transduction of the glucose-specific signal (Arenas-Huertero et al. 2000). Although, overexpression of *ABI5* has also been found to confer hypersensitive response to sugars, the *gin* phenotypes displayed by *abi5* alleles have not been found to be as strong as that of the *abi4* alleles (Huijser et al. 2000). *ABI4* locus has been found to encode a transcription factor of the APETALA2 (AP2) domain family that plays a major role during seed development and germination together with two other loci, *ABI3* and *ABI5* (Finkelstein 1994; Finkelstein et al. 1998), while the *ABI5* locus encodes a transcription factor that belongs to a large basic leucine zipper (bZIP) domain family which plays its role during late embryogenesis, in postgermination developmental arrest, and in specific tissues during vegetative growth. It has also been observed that important *cis*-acting sequences required for the regulation of *ABI4* gene lie at least 2-kb upstream of the start codon and the predicted amino acid sequence of ABI4 contains

a serine/threonine-rich domain, which is the possible target for protein kinases such as those of SnRK family. More importantly, ABI4 protein has been shown to mediate trehalose responses, to act as an essential element in the retrograde signaling (from plastids to the nucleus), and to regulate its own sugar-dependent expression (Koussevitzky et al. 2007; Ramon et al. 2007; Bossi et al. 2009). Moreover, both *ABI4* and *ABI5* are activated by glucose in an ABA-dependent fashion (Cheng et al. 2002). Extensive cross talk between sugar and ABA signaling pathways has been described for various aspects of plant growth and metabolism. On the one hand, sugars and ABA tend to act synergistically during embryo growth and storage reserve accumulation which is evident from the co-induction of sucrose induction of starch biosynthetic genes by ABA, while on the other hand, ABA and glucose has been found to act antagonistically during seed germination or early seedling growth, where exogenous glucose enables Arabidopsis seeds to germinate on otherwise inhibitory ABA concentrations (Eveland and Jackson 2012). α-Amylase transcript has also been reported to be induced by ABA (Ohto et al. 1992). Cho and Yoo (2011) have also reported a positive interaction between ABA and fructose signaling through hormone biosynthesis. Another link between the ABA and sugar-signaling pathway is supported by the observation that expression of GSQ5/DOG1 requires the ABA-mediated sugar-signaling pathway, whose alleles have been found to promote sugar induction of *ABI4* (Teng et al. 2008). Recently, a splicing factor SR45 has been identified as a negative regulator of sugar-signaling pathway and reported to be involved in the repression of glucose-induced ABA accumulation and downregulation of genes for ABA biosynthesis and signaling (Carvalho et al. 2010). The detailed analyses of sugar-insensitive mutants have suggested that ethylene and ABA act antagonistically in bringing the glucose response. The cross-linking interaction between these two hormones with respect to glucose has been further clarified by the analysis of double mutants *gin1 etr1* and *gin1 ein2*, which display the glucose-insensitive phenotype of the *gin1/aba2* mutant that clearly depicted that ethylene sensing and signaling pathways are tightly interconnected with those for sugar and ABA (Gazzarrini and McCourt 2001; Leon and Sheen 2003). Similarly, *gin1* mutant seedlings in *Arabidopsis*, overexpressing the *AtHXK1*, have been found to display glucose insensitivity, suggesting that *AtHXK1* acts upstream of ABA synthesis (Zhou et al. 1998) and FINS1 (fructose-1,6-bisphosphatse)-dependent fructose signaling has been found to act downstream of the abscisic acid pathway and interact positively with ABA signaling (Cho and Yoo 2011). FINS1 has been found to act downstream of *GIN1*, involved in ABA synthesis. These findings suggest that both fructose and glucose interact with ABA signaling with FINS1 and HXK1 function downstream and upstream of the ABA pathway, respectively. Further studies by Cheng et al. (2002) and Leon and Sheen (2003) have demonstrated that *GIN1/ABA2* encodes a short-chain dehydrogenase/reductase in ABA synthesis and that *CTR1/GIN4* encodes a putative mitogen-activated protein kinase kinase kinase which functions as a negative regulator of ethylene signaling, thereby suggesting that fructose signaling interacts positively with ABA signaling via hormone biosynthesis and interacts antagonistically with ethylene signaling via MAPKKK.

Cytokinins are another class of plant hormones found to regulate various processes including plant immunity (Barna et al. 2008). As far as their role in sugar sensing and signaling is concerned, they have been found to induce cell wall invertase (CWI) and hexose transporter expression in *Chenopodium rubrum* (Ehneb and Roitsch 1997). Cytokinins are known to delay senescence in plants and it has been speculated that they cannot delay leaf senescence in the absence of CWI activity (Balibrea-Lara et al. 2004). Invertases have been found to play an important role in sugar signaling by regulating the sucrose levels, sink strength, and sucrose: hexose ratio. Here the role of different invertases, including vacuolar, cell wall, and neutral/alkaline invertases, has been recognized (Xiang et al. 2011). De Coninck et al. (2005) have reported the existence of two forms of vacuolar invertase in

Arabidopsis (AtVI1 and AtVI2), both of which have been found to produce a rare sugar "1-kestose" in significant amounts under high sucrose concentration. A strong correlation between sucrose and cytokinin has been found to exist in the induction of the anthocyanin biosynthesis genes (Shan et al. 2009). Moreover, the effect of nitrate in the activation of maize photosynthesis gene expression has been proposed to be mediated through the elevation of cytokinins (Sakakibara et al. 1998).

Auxin is an important plant hormone that is a general regulator of growth and is also implicated in pattern formation, lateral organ development, and cell expansion (Kieffer et al. 2010). Various aspects of plant growth and development have been reported to be linked by sugar and auxin signals. The analyses of mutants like *hxk1/gin2* in *Arabidopsis* have revealed that these mutants on one hand are resistant to exogenous auxin and on the other hand are insensitive to high glucose (Moore et al. 2003). In *Arabidopsis* roots, the expression of a WUSCHEL (WUS)-related homeobox gene (*WOX5*), reported to maintain localized auxin in the root apical meristem, has been found to be induced by auxin and a nonmetabolizable sugar analog "turanose" (Gonzali et al. 2005). A substantial overlap of glucose and auxin response pathways has been reported by Mishra et al. (2009) in *Arabidopsis* seedlings in the control of root growth and development where about 62 % genes affected by auxin are transcriptionally regulated by glucose either antagonistically or synergistically. And more recently, in developing maize kernels, an auxin biosynthetic gene (*ZmYUCCA*) has been reported to be modulated by sugars thereby representing a link between sugar status and auxin signals (Le Clere et al. 2010). The conserved F-box and leucine-rich repeats between the glucose-regulated GRR1 in yeast and the auxin signaling component TIR1 in *Arabidopsis* also suggest another possible connection between glucose and auxin signaling (Ruegger et al. 1998). Interestingly, ethylene has also been suggested to play a role in root meristem maintenance through a mechanism that possibly involves auxin as has been evidenced by attenuation of ethylene effects in roots of certain auxin mutants (Ortega-Martinez et al. 2007) and ethylene-induced expression of auxin biosynthetic genes in the root meristem (Stepanova et al. 2008). However, it is not clearly determined whether sugar signals contribute to such signals or not, hence needs further elucidation.

Defense reactions of plants have also demonstrated an extensive cross talk between sugar and hormone signaling pathways (Leon and Sheen 2003). It has been suggested that plants react to pathogen invasion by production of some phytohormones which might function as signaling molecules for stimulation of plant innate immunity to activate defense responses (Pieterse et al. 2009). For that purpose, a fine-tuned cross talk among abscisic acid, jasmonate, salicylic acid, and PAMP-triggered signals has been shown to result in stomatal closure and then affects the defense responses together with other signaling pathways (Melotto et al. 2008; Ton et al. 2009; Cutler et al. 2010). In addition, many jasmonate and stress-inducible genes have been found to be coregulated by sugars (Reinbothe et al. 1994; Sadka et al. 1994). Jasmonic acid and a number of transcription factors have been recognized as potential regulators of the anthocyanin pathway in *Arabidopsis* (Gao et al. 2011; Qi et al. 2011), and it has also been shown that the cross talk among gibberellins, jasmonates, abscisic acid, and sucrose in a complex signaling network can modulate anthocyanin accumulation where sucrose signaling is regarded as a primary and essential component (Loreti et al. 2008). Similarly, the TATCCA element is also an important component of gibberellin response complex of the α-amylase gene in germinating cereal grains, suggesting the regulation of α-amylase gene expression by sugar and hormonal signal may share common regulatory mechanisms (Lu et al. 1998). In *Arabidopsis*, D-allose has also been reported to interfere with gibberellic acid (GA)-mediated signaling in a hexokinase-dependent way (Fukumoto et al. 2011). This underlines the central role of sugar-derived signals in plant growth, physiology, and development. However, many aspects of sugar-signaling pathways remain to be discovered and further studies will be required to reveal the genetic and molecular basis of sensing and signaling pathways connecting sugar and hormonal regulation in plants.

5.5 Conclusion

During the last decade, a significant progress has been made in elucidating the role of sugars or their analogs in diverse signal transduction pathways in plants and other organisms. It is the result of tireless efforts by the researchers that led to identification of sugars as signaling molecules, earlier thought to be only simple metabolites and energy sources. Sugar sensing has been implicated in a wide range of metabolic activities related to plant growth and development ranging from seed germination to seedling development and root/leaf differentiation or flower formation to senescence and in regulating responses to light, stress, and pathogens, through a complicated network of signaling cascades involving diverse signaling components. The research progress made in the past few years has also made it clear that different sugars (glucose, fructose, sucrose, mannose, etc.) act as distinct signals suggesting the involvement of multiple sugar sensors/receptors in mediating parallel signaling pathways. Different sugar sensors have been identified and found to coordinate various developmental processes involving at least three distinct pathways: hexokinase-dependent/metabolism-dependent, hexokinase-dependent/metabolism-independent, and hexokinase-independent pathways. These signal transduction pathways involve their respective sensors and the downstream components involving hexokinases, fructokinases, sucrose transporters, G proteins, protein kinases, protein phosphatases, cyclic nucleotides (cAMP), Ca^{2+}/calmodulin, and a range of transcription factors to regulate gene expression. Although a detailed study of hexokinase-dependent pathway has been made, the hexokinase-independent pathway has not been fully characterized, and here the combination of molecular, biochemical, and genetic approaches promises to unravel more detailed mechanisms underlying it. The role of trehalose in defense responses of plants has also been demonstrated but the exact signaling nature and the transduction pathway of trehalose is not clear as yet. The analyses of sugar-signaling mutants in *Arabidopsis* have also demonstrated an extensive and complicated cross talk between sugar and hormone signaling pathways particularly between glucose and abscisic acid signaling. Similar crosstalk between sugars and other hormones is also emerging but needs further elucidation at the molecular or biochemical level.

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6 Nitrogen Regulation and Signalling in Plants

Khalid Rehman Hakeem, Muhammad Sabir, Faheema Khan, and Reiaz Ul Rehman

Abstract

Nitrogen (N) is an essentially critical element which is involved in signalling and can affect plant growth and development. The plants have evolved different strategies involving short- and long-ranged signalling pathways to cope with the changes in N regulations in the soil. These pathways work at cell and plant level for coordination of N metabolism, growth, and development in accordance with the external and the internal N status. Currently, identification and characterization of local and systemic signalling has been emphasized, but information about integrating coordination and organization of N response of the plants is still lacking. Tracing out the full N pathway could help us to devise and produce high N-efficient genotypes and increase Nitrogen use efficiency (NUE). In this chapter, we discuss the physiological as well as molecular means to understand the mechanisms involved in local and systemic nitrogen responses and how these responses are coordinated.

Keywords

Nitrogen • Signal molecule • N-metabolic pathway • N regulation • NUE

K.R. Hakeem (✉)
Faculty of Forestry, Universiti Putra Malaysia, Serdang 43400, Selangor, Malaysia
e-mail: kur.hakeem@gmail.com

M. Sabir
Institute of Soil and Environmental Sciences, University of Agriculture, Faisalabad 38040, Pakistan
e-mail: cmsuaf@gmail.com

F. Khan
Department of Botany and Microbiology, King Saud University, Riyadh, Saudi Arabia
e-mail: drfaheemakhan@gmail.com

R. Ul Rehman
Department of Bioresources, University of Kashmir, Srinagar 190006, Jammu and Kashmir, India
e-mail: rreiazbiores@gmail.com

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6.1 Introduction

Nitrogen (N) is a macronutrient required for the normal plant growth and development. Nitrogen inputs actually define the yield and productivity of crops (Hakeem et al. 2011, 2012a). It has been estimated that the use of nitrogenous fertilizers has increased sevenfold which has doubled food production during the last century. Globally, demand for nitrogenous fertilizer has increased tremendously during the last century, and it will continue to rise at the rate of 1 % per annum (FAO 2007). Nitrogen use efficiency (NUE), the grain yield per unit of nitrogen available from the soil, including the residual N present in the soil and the fertilizers (Moll et al. 1982), under field conditions is about 30–40 %, and the remaining N is lost into the environment particularly in the intensive agriculture. The remaining 60–70 % of unused nitrogen (N) creates severe environmental as well as health problems. The unused nitrate leaches to ground water and causes several health effects, such as methemoglobinemia and gastric cancer, among others (Hakeem et al. 2011). Emission of unused N, from agricultural fields, in the form of NO_x contributes to global warming and other harmful effects to the environment (Hakeem et al. 2012b). The Delhi Declaration of the International Nitrogen Initiative (http://initrogen.org/index.php/publications/delhi-declaration) showed the concern about rising N losses from agriculture and insisted for devising a comprehensive strategy for efficient N management in agricultural and industrial sectors. Nitrogen use efficiency can be enhanced by optimizing interaction between fertilizer soil and water.

It is evident that plants use different strategies in response to the variations in N supply (e.g., localized NO_3^- in the soil, uniform high NO_3^-, and N starvation). The sensing and signalling for deprivation of nutrients are not well characterized particularly for N deprivation. Signal transduction cascade involved in phosphorus-deprivation response has been identified which is controlled by sumoylation. Two microRNAs control the gene expression under P and S deficiency. Understanding of short-term and longer-term responses is necessary for progression of signalling events under limited internal and external supply of nutritions.

In this chapter we tried to gather the latest information about the nitrogen metabolism and its regulation, also to trace out its signalling pathway.

6.2 Nitrogen: An Important Plant Nutrient

Mineral elements play an important role in growth and development of plants. There are 17 essential elements, of which six are required in large quantities. During the past half century, supply of mineral nutrients, especially N, P, and K, through fertilizers has been considered as an important input in agriculture sector for crop production. Nitrogen occupies a unique position among these mineral elements since it is an important component of different organic compounds and structures in plant cells. Nitrogen has a role in energy transfer by being part of compounds, like ATP (adenosine triphosphate) which is responsible for conservation and use of energy during metabolism by the cells. It is a component of nucleic acids (DNA, RNA), proteins, vitamins, and hormones. Nitrogen supply determines the nature and diversity of plants, the population of grazing animals and their predators, plant productivity, and the cycling of carbon and soil minerals in many terrestrial and aquatic systems (CFAITC 2008). In agriculture, application of nitrogen influences yield and quality of crops. Thus, it has greatly influence food security, economic development, and environmental quality. Nitrogen deficiencies lead to chlorosis and reduced photosynthesis which ultimately result in lower yields. The deficiency of N causes a significant reduction in the levels of phosphoenolpyruvate carboxylase, pyruvate orthophosphate dikinase, and Rubisco and a concomitant decrease in the level of their respective mRNAs in developing maize leaves (Khamis et al. 1990). Proteomics studies also revealed that proteins like glutamine synthetase, phytoene synthase,

enoyl-CoA hydratase, 2-cys peroxiredoxin BAS 1, and enolase-2 protein were involved in conferring NUE to the N-efficient rice cultivars/genotypes (Hakeem et al. 2012a).

6.3 Nitrogen in Plants

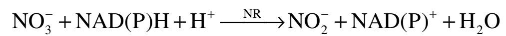
Nitrogen exists in the form of molecular N_2, volatile ammonia, oxides of N, NO_3^-, NH_4^+, and organic N (urea and amino acids). However, NO_3^- and ammonium are the preferred source of N by most plant species (Leleu and Vuylsteker 2004; Hakeem et al. 2011). Under aerobic soil conditions, although ammonium is also present but nitrate is the dominant N form. It is the most abundant which is under cultivation of annual crops. Generally, field crops prefer a mixture of NH_4^+ and NO_3^-, but proportionally uptake of ammonium is more than NO_3^- compared to their proportion in the soil solution. It was reported after the analysis of 35 soil samples from agricultural fields that NO_3^- in soil solution was 6.0 mM compared to 0.77 mM of NH_4^+ (Wolt 1994). Uptake and assimilation of N are described in the following sections.

6.4 Nitrogen Uptake

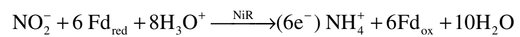
The first step in N acquisition and its use by plants is the nitrate (NO_3^-) transport from soil through plasmalemma of epidermal and cortical cells of the root (Miller and Cramer 2004). According to Forde (2007), NO_3^- can directly affect the gene expression associated with NO_3^- uptake, transport, and assimilation. It thus acts as both a nutrient and a signal. Although most of the higher plants can reduce NO_3^- in roots and shoots, the reduction of NO_3^- is more efficient in leaves than in roots due to easy availability of reductants, energy, and carbon skeletons produced by photosynthesis (Chen et al. 2004). NO_3^- uptake is an energy-dependent process triggered by the gradient of proton or the proton-motive force maintained by H^+-ATPase. Two or more protons are cotransported along with every NO_3^- ion (Santi et al. 1995). In many higher plants, there exists a biphasic relationship between NO_3^- uptake rate and external NO_3^- concentration. It is suggested that there are two different types of transporter systems in higher plants, namely, high-affinity transporter system (HATS) and low-affinity transporter system (LATS). HATS are further categorized into *inducible high-affinity transporter system (iHATS)* and *constitutive high-affinity transporter system (cHATS)* (Okamoto et al. 2006).

6.5 Nitrogen Assimilation

Once the NO_3^- has been taken up by the plant cell, the next step in the N-assimilation pathway is the reduction of NO_3^-. The NO_3^- entering the plant cell is assimilated in a series of steps involving the action of a number of different enzymes (Crawford et al. 2000). This reduction is catalyzed by NO_3^- reductase (NR) and nitrite reductase (NiR). NR reduces the NO_3^- into nitrite, which is then reduced to ammonium by NiR.

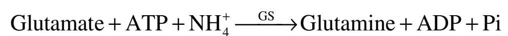


$$NO_3^- + NAD(P)H + H^+ \xrightarrow{NR} NO_2^- + NAD(P)^+ + H_2O$$



$$NO_2^- + 6\,Fd_{red} + 8H_3O^+ \xrightarrow{NiR} (6e^-)\,NH_4^+ + 6Fd_{ox} + 10H_2O$$

In plants, NH_4^+ formed after reduction of NO_3^- in the plant or absorbed directly from soils is rapidly converted into nontoxic organic compounds. In plants, NH_4^+ assimilation to synthesize amino acids is mediated by two enzymes: glutamine synthetase (GS) and glutamate synthase (GOGAT). GS catalyzes the first step of NH_4^+ assimilation into glutamate for synthesis of glutamine (Suzuki and Knaff 2005). The reaction is as follows:



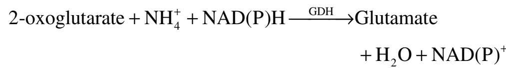
$$Glutamate + ATP + NH_4^+ \xrightarrow{GS} Glutamine + ADP + Pi$$

GOGAT catalyzes the synthesis of two molecules of glutamate. The reaction is as follows:

$$Glutamine + 2\text{-oxoglutarate} \xrightarrow{GOGAT} 2Glutamate$$

GS/GOGAT pathway is important as glutamine and glutamate synthesized produced serve as donors of amino groups for the amino acid biosynthesis, nucleotides, chlorophylls, polyamines, and alkaloids (Hirel and Lea 2001).

Alternatively, glutamate formation involves reductive amination of 2-oxoglutarate by NH_4^+, catalyzed by mitochondrial glutamate dehydrogenase (GDH). The reaction is as follows:



$$\text{2-oxoglutarate} + NH_4^+ + NAD(P)H \xrightarrow{GDH} \text{Glutamate} + H_2O + NAD(P)^+$$

6.6 Molecular Physiology of Nitrate Uptake

6.6.1 Biochemical Characterization

Inducible high-affinity transporter system (iHATS) is substrate inducible and is responsible for uptake at low concentrations of NO_3^- (below ~1 mM) that are characterized by low K_m values (5–200 μM). Constitutive high-affinity transporter system (cHATS) is responsible for uptake at low concentrations of NO_3^- and provides a low-capacity pathway in uninduced plants but operates simultaneously with *iHATS* in the induced state. Their activity becomes threefold on exposure to NO_3^- (Crawford and Glass 1998). It is characterized by low values of both K_m (6–20 μM) and V_{max}. Low-affinity transporters form a constitutive transport system which is responsible for uptake at high external NO_3^- concentrations (>1 mM). Despite of showing linear kinetics, it is a transport system which is an active H^+-dependent system (Kronzucker et al. 1995). LATS are characterized by high K_m values (>0.5 mM). It allows enough NO_3^- into the cell which is sufficient to cause the expression of transporter and genes and probably plays a physiological role in the NO_3^- uptake only above a certain threshold. Molecular studies of NO_3^- transporters in plant suggest that NO_3^- transporters belong to two different families, NNP and PTR (Forde 2000).

It is suggested that high- and low-affinity transporters are functional at early growth stages but the high-affinity transporters are also functional at later stages when the soil N concentration is low. Studies on oilseed rape showed that HATS accounted for about 89 % of the total NO_3^- uptake (18 and 79 % for cHATS and iHATS, respectively) when no fertilizer was applied (Malagoli et al. 2004). They also found that LATS accounted for a minor proportion of the total NO_3^- uptake. It is suggested that NO_3^--inducible part of HATS functions mainly as a sensor for NO_3^- availability to the roots (Miller et al. 2007a).

6.6.2 Genomic Organization

Nrt1 and *Nrt2* gene families characterize two classes of membrane proteins which might involve in low- and high-affinity NO_3^- transport, respectively (Okamoto et al. 2003). Unkles et al. (1991) isolated the first eukaryotic NO_3^- transporter gene from the fungus, *Aspergillus nidulans*. Later on, a NO_3^- transporter gene (*AtNrt1:1)* was identified in *Arabidopsis thaliana* (Tsay et al. 1993). Later, this gene was used as a probe to isolate two more genes (*LeNrt1:1)* and (*LeNrt1:2)* in tomato (Lauter et al. 1996). Here, *Nrt1:2* is shown to be NO_3^- inducible and its expression restricted to roots except in stem or leaves, but *Nrt1:1* is not limited to roots and is constitutively expressed. Nitrate transporter genes have been cloned from wide range of plants. *BnNrt1:2* is identified in *Brassica napus* also (Crawford and Glass 1998). *AtNrt1:4* is expressed in a specific pattern petiole of leaf and plays an important role during accumulation of NO_3^- in these tissues (Chiu et al. 2004). *AtNrt1:3* expression was NO_3^- induced in the leaf, but in roots it contributed significantly to LATS (Okamoto et al. 2003). According to Li et al. (2007), *AtNrt2:1* was the main contributor to iHATS and cHATS.

Ammonium (NH_4^+) transport shows a normal homeostatic tendency, but the range of the concentration at which absorption occurs is very limited due to the potential toxicity at elevated NH_4^+ concentrations. Like NO_3^-, ammonium is also transported by transporter protein located in the plasma membrane. AMT-type transporters handle NH_4^+ influx and mediate the uniport of this ion. First NH_4^+ transporter *AtAMT1;1* was isolated from *Arabidopsis thaliana* (Ninnemann et al. 1994) and then another five homologous sequences, *AtAMT1;1* to *AtAMT1;5* were discovered.

6.6.3 Regulation of Transporters

Signals derived from NO_3^- for regulating NO_3^- uptake trigger changes in gene expression which cause N-metabolism reprogramming for facilitating the absorption and incorporation of NO_3^- and amino acid synthesis. Available NO_3^- and reduced N regulates the assimilatory pathway of NO_3^-. It was reported that increasing concentration of NO_3^- increased the NO_3^- uptake in strawberry (Taghavi and Babalar 2007). Many LATS- and HATS-related genes are inducible by NO_3^-, and one HATS-related gene *NpNrt2:1* is also repressible for reduced N (Quesada et al. 1997). The cHATS gives a high affinity having less capacity pathway for nitrate entry into uninduced barley and white spruce plants. Nevertheless, activity of cHATS is controlled by exposure to NO_3^- by three times (Trueman et al. 1996). In barley, the fully induced iHATS flux was about 30 times higher than that resulted from the cHATS (Quesada et al. 1997). It has been observed that increasing transcription increases NO_3^- absorption rate (Imsande and Touraine 1994). It is reported that in citrus seedlings, LATS is controlled by feedback mechanism induced by the N contents of plant. The absorption of NO_3^- is reported to decrease due to the application of amino acids (Glu, Asp, Asn, Gln) to the external solution (Cerezo et al. 2000). The importance of biosynthesis of proteins for NO_3^- uptake is evident because of using of chemical inhibitors in physiological studies (Aguera et al. 1990). Cerezo et al. (2001) proposed mechanism of degradation for transporter protein in Arabidopsis. The presence of conserved protein kinase carbon recognition motifs in the nitrogen and carbon domains of *HvNRT2:1* (Forde 2000) indicated that phosphorylation activities are involved in regulation of *AtNrt2:1* activity in response to different factors of environment. Remans et al. (2006) reported that *AtNrt2:1* played an important role as a major NO_3^- absorption under N-deficient conditions and influenced initiation and development of lateral root system with external NO_3^- availability.

6.6.4 Regulation of Nitrogen Metabolism

Competition for N resources by crops causes nutrient deficiency in the soil during the interval between fertilizations. Nitrogen nutrition index (NNI) is used for assessment of sub- versus supraoptimal N supply in agriculture (Richard-Molard et al. 2008). Study of these processes helps environmentally safe production of the crops which is the demand of sustainable agriculture. New bioinformatics tools (sun gear software) can be used nowadays for detailed study of NO_3^- response in plants. These analyses have identified many genes and pathways (Gutierrez et al. 2007). One might think that NO_3^- is used in a linear pathway involving absorption and transport, followed by its assimilation, and biosynthesis of amino acids and protein. But, complex interactions including storage and remobilization of NO_3^-, assimilation of de novo NH_4^+, and distribution of N in highly branched pathway of amino acid synthesis are affected by temporal changes in expression of gene, enzymatic activities, metabolite levels, and fluxes. Leaves contained high contents of NIA transcription at the end of night when grown in high NO_3^- and favorable light conditions (Kaiser et al. 1999) which led to around three times increase during initial hours of exposure to light (Scheible et al. 1997). The NO_3^- assimilation rate surpasses net flux through GOGAT pathway by 25 % and results in assimilation of reduced N in products such as NH_4^+ and glutamine, photorespiratory metabolites, glycine, and serine. Usually, assimilation of NO_3^- and NH_4^+ is controlled through transcriptions due to the balance between NO_3^- influx and its incorporation and posttranscriptionally due to the signals from nitrogen metabolism. Increase of NH_4^+ and glutamine on feeding glutamate indicates the important role of glutamate in the sensitive feedback mechanism responsible for regulation of NH_4^+ assimilation. Concentration of amino acids in plants regulates the NO_3^- uptake by plants by indicating N status by signalling (Miller et al. 2007b). It is reported that acids, including cysteine and asparagine, play an important role in feedback regulation on NO_3^-

assimilation. Glutamate, cysteine, and asparagine have important positions in metabolism of amino acid for feedback inhibitors of NO_3^- reduction.

6.7 Molecular Physiology of N-Assimilatory Enzymes

6.7.1 Nitrate Reductase

6.7.1.1 Biochemical Characterization and Localization

Nitrate reductase (NR) is the most important enzyme which catalyzes the first step of NO_3^- assimilation in plants (Leleu and Vuylsteker 2004). It reduces NO_3^- to nitrite with pyrimidine nucleotide in higher plants. This is a soluble enzyme in plants that is present in the cytosols of epidermal cells of roots and cortical cells and mesophyll cells of shoot. It exists as a homodimer metalloprotein of 110-kD subunits. NO_3^- reduction to nitrite is catalyzed by NR by transferring two electrons from NAD(P)H to NO_3^- via three redox centers composed of two prosthetic groups (flavin adenine dinucleotide [FAD] and heme) and a MoCo cofactor in a 1:1:1 stoichiometry per subunit. Each redox center is attached to an independent functional domain of the enzyme having activity unaffected by other domains. NR is a substrate-inducible enzyme which is the most important limiting step in N assimilation. For this reason, NR activity is considered as a selection criterion for grain yield and N-assimilation potential. Nitrate reductase in plants exists in three major forms which are characterized by electron-donor source, either NADH and/or NADPH (Miller and Cramer 2004).

6.7.1.2 Genomic Organization and Regulation

NO_3^- reductase is regulated in both shoots and roots. Most plants have two or more genes for NR. Clones of both genes have been isolated and mapped (Sivasankar and Oaks 1995). Molecular and genetic analyses have indicated that in plants there are two or more structural genes for NR, the only known exception being *Nicotiana plumbaginifolia*, that has a single gene for NR, that encodes an NADH-dependent NR (Caboche and Rouze 1990). In barley, the NADH-specific NR is encoded by *nar*I gene, while the NADPH-bispecific NR is encoded by *nar*7 gene. Clones of both genes have been isolated and mapped (Miyazaki et al. 1991) and their induction properties have been compared (Sueyoshi et al. 1995). Although, the two proteins are distinct, the genes respond similarly to NO_3^-.

NR activity can be controlled either by modifying the activity of already present enzyme or by maintaining the amount of enzyme by producing new enzymes and degradation of the old one. Many factors regulate this enzyme. NO_3^- triggers transcription of inducible genes (NIA) encoding NR. De novo synthesis of new NR, stimulated by NO_3^-, is one of the mechanisms for controlling enzyme level when combined with NR-protein degradation (Stitt 1999). NO_3^--induced increase in the NR activity and NR protein is due to the enhanced steady state level of NR-mRNA (Miyazaki et al. 1991; Sueyoshi et al. 1995), and this induction is shown to be repressed by downstream N-assimilation products like glutamine and asparagine (Vincentz et al. 1993; Sivasankar et al. 1997). Sucrose addition (Sivasankar and Oaks 1995) and light also enhance NR protein and mRNA induction. It appears that NR expression is regulated by light via phytochrome after it is triggered by NO_3^-. The role of light in induction is probably more related to the activity of the enzyme rather than to the activation of the NR gene. In another study, NR-protein kinase was used to identify the apparent key serine residue in spinach NR (Bachmann et al. 1996).

Many environmental factors initiate the modulation and posttranslational regulation of NR. The total amount of NO_3^- reductase depends simultaneously on its synthesis and degradation. Activity level can be controlled by mechanisms involving phosphorylation of the NR protein and binding of Mg^{2+} or other divalent cation and an inhibitor protein (Stitt 1999). Light has a very important effect though it is not a direct signal for activation of NR, but photosynthetic process is needed for activation of NR. It has been reported that the availability of oxygen and light is the important external factor for rapid and reversible

modulation of NR activity, and feeding of sugar in the leaves in darkness activated the NR. Sugar and/or phosphates of sugars are the internal signals regulating the protein kinase(s) and phosphatase. The roots usually do not change their reduction rate as rapidly as shoots. However, during sudden anoxia, the enzyme is rapidly modulated, being activated within minutes (Kaiser and Huber 2001).

6.7.2 Nitrite Reductase (EC 1.7.7.1)

6.7.2.1 Biochemical Characterization and Localization

The second enzyme in the sequence, nitrite reductase (NiR, ferredoxin nitrite oxidoreductase), catalyzes the six-electron transfer reaction from reduced ferredoxin to NO_3^-, leading to the synthesis of NH_4^+. It is localized in chloroplasts in leaf and in plastids of root cells (Sechley et al. 1992). It is a monomeric protein of about 63 kDa containing sirohaem and a 4Fe4S center as prosthetic groups (Faure et al. 2001). Reduced ferredoxin acts as the electron donor in both leaves and roots. This enzyme gains reducing power from NADPH, produced by the oxidative pentose phosphate pathway present in the plastids of the roots (Bowsher et al. 1989). It is reported that ferredoxin and the NADPH-dependent ferredoxin:NADP-oxidoreductase increase in isolated pea root plastids due to NO_3^- application.

6.7.2.2 Genomic Organization and Regulation

The gene for the NiR apoprotein has been cloned in at least six different plant species. There is one NiR apoprotein gene per haploid genome in barley and spinach, two in maize, and four in *N. tabacum* (Kronenberger et al. 1993). The four NiR apoprotein genes in tobacco are reported to be involved in encoding of two different isoforms in shoots and in roots, as evidenced by the gene expression (Kronenberger et al. 1993). The promoter region of NiR gene has been fused to β-glucuronidase (GUS), and transgene has then been successfully introduced into tobacco (Rastogi et al. 1993).

This gene appears to be very responsive to NO_3^- additions and to the additions of sucrose, glutamine, or asparagine. The experiments of Rastogi et al. (1993) provide evidence that induction of this gene by NO_3^- is a transcriptional event. However, the addition of asparagine or glutamine results in a repression of induction, whereas sucrose enhances the induction (Sivasankar and Oaks 1995). Light is also an important environmental cue in the NiR induction (Wray 1993).

6.7.3 Glutamine Synthetase (EC 6.3.1.2)

6.7.3.1 Biochemical Characterization and Localization

Glutamine synthetase (GS) mediates the ATP-dependent transformation of inorganic N (NH_4^+) to an organic form like glutamine. This enzyme along with GOGAT represents the major pathway for incorporation of ammonia (toxic to plant function) into amino acids (Hirel and Lea 2001; Fei et al. 2006). There are two types of GS: type-I GS, which is dodecameric with subunits of about 52 kDa, and type-II GS that is octameric and composed of about 40 kDa subunits (Nogueira et al. 2005). Type I is found mainly in bacteria and type II is best identified in higher plants. In plants, this is present as a chloroplastic (GS2) and a cytosolic (GS1) enzyme (Scarpeci et al. 2007). Nitrogen, which is moved through a small number of transport compounds, is added to and removed from proteins in different organs during different stages of plant development. A major portion of N is released as ammonia and reassimilated via GS (Miflin and Habash 2002).

6.7.3.2 Genomic Organization and Regulation

Molecular analysis of GS genes reveals a multigene family whose individual members encode several distinct cytosolic GS (GS1) polypeptides and a single chloroplastic GS (GS2) polypeptide. Li et al. (1993) have identified five distinct cDNA clones of GS in maize. Six distinct genes encoding for GS in maize (Li et al. 1993) and five in sugarcane (Nogueira et al. 2005) have been identified. It has been demonstrated that GS occurs in an organ-specific manner; roots and

nodules generally contain proportionally more cytosolic GS, while leaves contain more chloroplastic GS (Becker et al. 1992). Genetic study of GS has helped in explicating the role of each isoform. Chloroplastic GS is considered to be involved in the reassimilation of photorespiratory NH_4^+.

The genes GS2 and GS1 are controlled by the external N application, but the extent of control is a plant species dependent, N source, and plant organ/tissue (Cren and Hirel 1999). Nitrogen assimilation whether in the form of NH_4^+ and/or NO_3^- has a regulatory effect on gene expression in rice, maize, tobacco, tomato, sunflower, and mustard (Zozaya-Hinchliffe et al. 2005). Nitrogen and carbon metabolites could also control the GS expression in the leaf of Arabidopsis (Oliveira and Coruzzi 1999) and tobacco (Masclaux-Daubresse et al. 2005). According to Zozaya-Hinchliffe et al. (2005), light and metabolic factors associated with light also regulate the expression of this enzyme. Detailed studies on *Pinus sylvestris* by Elmlinger et al. (1994) have shown that regulation of GS2 expression through light occurs crudely at transcriptional level, but fine regulation occurs at the posttranscriptional level. Similar observations were made in seedlings of tomato (Migge et al. 1998). Some mechanisms controlling the stability and activity of GS have been identified. Finnermann and Schjoerring (2000) proposed a model that can control GS1 reversibly by phosphorylation and dephosphorylation which incorporated the roles of ATP, Mg^{2+}, and 14-3-3 binding. This model is based on the major role of ATP/AMP ratio in the light where it is suggested that levels of ATP/AMP are high in dark, and therefore GS1 is phosphorylated and binds 14-3-3 proteins to protect its deterioration. Conversely, in the light, GS1 is unphosphorylated and becomes susceptible to damage. Riedel et al. (2001) have also demonstrated that GS2 is phosphorylated in tobacco. Many workers have shown that important factors affecting GS activity are light, C status, and N nutrition. Experiments with white, red, far-red, or blue light by Becker et al. (1992) and Migge et al. (1998) have shown that the phytochrome and the blue light photoreceptors are responsible for positive response to light. Carbon compounds, important in the stimulation of GS1 and GS2 production, include sucrose and 2-oxoglutarate (Oliveira et al. 2002). Studies on dark-adapted *Arabidopsis* seedlings have shown that sucrose enhances the expression of GS2, thus representing the light effect. Woodall et al. (1996) conducted experiments on pea and barley plants and concluded that temperature is a crucial factor that controls the GS expression. Within 2 days of keeping the plants in 15 °C instead of 25 °C, they observed 50 % reduction in GS2 activity, while GS1 activity remained unaffected. There are indications that substrate availability (Ortega et al. 1999) or phosphorylation (Moorhead et al. 1999) may be an important factor controlling the enzyme turnover and activity respectively.

6.7.4 Glutamate Synthase (E.C. 1.4.1.13)

6.7.4.1 Biochemical Characterization and Localization

Glutamate synthase (Glutamine (amide):2-oxoglutarate aminotransferase, GOGAT) mediates the transfer of the amide group of glutamine (produced by GS) to 2-oxoglutarate (α-keto glutarate) to form two glutamate molecules through reduction (Ireland and Lea 1999). The discovery of NAD(P)H-dependent GOGAT in bacteria (Tempest et al. 1970), ferredoxin (Fd)-dependent GOGAT in pea chloroplast (Lea and Miflin 1974), and NAD(P)H-dependent GOGAT in carrot cell cultures (Dougall 1974) established a route, GS-GOGAT cycle, for the incorporation of NH_3 into organic compounds. The synthesized glutamate may be utilized either to fulfill the glutamate pool for subsequent GS catalysis or to donate its amino group to form other N-containing compounds. One important fate of glutamate and glutamine is the synthesis of aspartate and asparagine. These amino acids are important N-transport compounds in many plants (Temple et al. 1998). In higher plants, GOGAT are present as two different isoforms, NADH-GOGAT (EC 1.4.1.14) and Fd-GOGAT (EC 1.2.7.1), and these differ in molecular mass, subunit composition,

enzyme kinetics, and metabolic functions (Gregerson et al. 1993; Sakakibara et al. 1991). Fd-GOGAT, an iron-sulfur flavoprotein, generally functions as a monomer with subunit molecular mass of 130–180 kD. The presence of Fd-GOGAT isoform in maize roots is different from the enzyme present in leaves, and this suggested that these are encoded by separate genes. The root isoform has been involved in NH_4^+ assimilation which is derived from soil NO_3^- (Redinbaugh and Campbell 1993). NADH-GOGAT is also an iron-sulfur flavoprotein and is found primarily in nongreen tissues. In higher plants, it occurs as a monomer with a native subunit mass of 225–230 kDa and has a pH-optimum range from 7.5 to 8.5 (Lea et al. 1990).

6.7.4.2 Genomic Organization and Regulation

GOGAT is found in all types of organism, and its amino acid sequence is remarkably well conserved (Temple et al. 1998). The expression pattern of the genes encoding cytosolic GS and NADH-GOGAT appears to be synchronized in nonlegumes where the function of proteins coupled with processes such as primary NH_4^+ assimilation derived from soil nitrate and reassimilation of ammonium released due to catabolism of amino acid. cDNA clones for Fd-GOGAT have been isolated from a number of species including barley (Avila et al. 1993), maize (Sakakibara et al. 1991), and *A. thaliana* (Coschigano et al. 1998). Full-length cDNA and genomic clones of NADH-GOGAT have been isolated from alfalfa (Trepp et al. 1999) and rice (Goto et al. 1998).

Light and a variety of metabolites exert major regulatory controls over metabolic pathways. Evidence by Suzuki and Rothstein (1997) indicates that light exerts a positive regulatory effect on the expression of Fd-GOGAT (GLU1). GLU2 expression is also induced by light, but the induction of this gene by sucrose in dark indicates that light-induced expression may in part be caused by increased concentration of C metabolites. The Fd-GOGAT activity increases with the start of photosynthesis and photorespiration during the expansion and development of a new leaf (Emes and Tobin 1993). In barley, enzymatic activity, mRNA, and protein increased on the leaf emergence and expansion and decreased aging of the leaf (Pajuelo et al. 1997). Nitrate also acts as a signal resulting in wide range changes in the expression of key genes in N-metabolism pathway, including Fd-GOGAT (Scheible et al. 1997). Gene expression study in developing alfalfa nodules suggests that NADH-GOGAT is uniquely regulated, as compared with other genes of N metabolism (Vance et al. 1994). The expression of NADH-GOGAT occurred in effective nodules and in ineffective nodules, and roots was only 12–20 % of the maximum. These results show that active N fixation and NH_4^+ itself or a downstream product of its metabolism is required for maximum NADH-GOGAT gene expression. Fd-GOGAT proteins from *Arabidopsis* and maize contain a presequence with many of characteristics of plastid transit peptides. Similarly, both rice and alfalfa (Gregerson et al. 1993) NADH-GOGATs contain presequences that are thought to be responsible for targeting of plastids. Interestingly, presequences are found in all characterized eukaryotic GOGAT proteins. As shown by the experiments of Yamaya (2003), NH_4^+ ions and glutamine could serve as signals for the transcription increase.

6.7.5 Glutamate Dehydrogenase (EC 1.4.1.2)

6.7.5.1 Biochemical Characterization and Localization

Glutamate dehydrogenase (GDH) can release N from amino acids to generate keto acid and ammonia which can be recycled separately for use in respiration and amide formation, respectively. It is thought to be an alternative pathway for the glutamate formation involving reductive amination of 2-oxoglutarate by NH_4^+. Its role in plant cells remains controversial (Miflin and Habash 2002). It is yet to be clearly established that the enzyme has an important role in NH_3 assimilation or in recycling of carbon (Dubois et al. 2003). Studies have shown that it is involved

in the deamination of glutamate for energy supply and return carbon from amino acids into metabolism of carbon during shortage of carbon or energy (Miflin and Habash 2002). However, Dubois et al. (2003) have still speculated the physiological role of GDH in plants. GDH is capable of synthesizing or deaminating glutamate, but the direction of activity depends on specific environmental cues (Pahlich 1996). One isoform of enzyme, localized in mitochondria in roots and leaves, uses NADH as the electron donor (Sechley et al. 1992). Another isoform, having specific requirement for NADPH, is present in chloroplasts of photosynthetic tissues. The primary role of GDH could be replenishment of TCA cycle intermediates via its oxidation to 2-oxoglutarate. Glutamate is deaminated to 2-oxoglutarate in isolated mitochondria; however, in the presence of aminooxyacetate, glutamate no longer contribute to mitochondrial respiration (Sechley et al. 1992). This observation indicates that GDH does not oxidize glutamate.

6.8 Improving NUE Through Manipulation of Signalling Targets

The failure to improve NUE in transgenic plants by overexpression of enzymes of NO_3^- and NH_3 incorporation has supported the theory that metabolic flux via such pathways may be regulated by regulatory switches outside these pathways. The NO_3^- sensing and signalling is not well understood. Posttranslational control of some nitrate-responsive enzymes is carried out by 14-3-3 proteins despite the fact that these control light effect and other signals, rather than NO_3^-. Some elements which could be related with NO_3^- signalling are Ca^{2+} and protein kinases/phosphatases. These elements are involved in mediation of the NO_3^- signal which is responsible for expressing NR, NiR, and GS2 mRNAs (Sakakibara et al. 1992; Sueyoshi et al. 1999). Hartwell et al. (1999) observed that Ca^{2+}-free PEPCase protein kinase is a member of the Ca^{2+} calmodulin-regulated group of protein kinases. Krapp et al. (2002) explained the specific roles of these groups in mediation of NO_3^- and other interacting signals. Study of mutants associated with signal-transfer cascade can lead to better understanding of the nitrate-signalling cascade from NO_3^- to the NR gene (Ogawa et al. 2000). It reveals some intermediary and potential sites for the management of NUE. Light is an another signal which is responsible for the regulation of the expression of many nitrate-responsive genes (Raghuram and Sopory 1995; Chandok et al. 1997; Lillo and Appenroth 2001). In green plants, light controls signalling and NUE via the photosynthetic process and sugars (Lillo and Appenroth 2001). At the posttranslational level, light modulates the enzyme phosphorylation status, in connection with 14-3-3 proteins.

Transcription of NO_3^--responsive genes by NO_3^- as a signal needs cis-acting regulatory sequences or NO_3^--responsive elements (Raghuram et al. 2006). Zhang and Forde (1998) reported the presence of ANR1, a putative signalling and NUE transcriptional factor which is similar to the MADS-box family in *Arabidopsis thaliana*. ANR1 is NO_3^- inducible and root specific which is responsible for lateral-root proliferation due to NO_3^- in transgenic plants (Forde 2007). However, this type of transcription is not related with known NO_3^- responses even in the root. The manipulation of N by Dof1 overexpression shows that understanding of the signalling mechanisms may lead to new goals and techniques for metabolic-engineering activities in future (Lochab et al. 2007). Yanagisawa et al. (2004) developed such transgenic *Arabidopsis* lines that can overexpress Dof1. Dof1 is a maize protein of Dof family of plant-specific transcriptional factors which is responsible for activation of different genes responsible for C-metabolism related to metabolism of organic acid. The transformants indicated approximately 30 % more mRNA and enzymatic activities for PEP carboxylase and pyruvate kinase, without decreasing GS, NR, and GOGAT RNAs. If Dof1 is not induced by NO_3^-, it will indicate numerous transcriptional factors which may mediate in the coordination of gene expression responsible for nitrogen and carbon metabolism.

6.9 Understanding Nitrogen Regulation Through Proteomics

The complete genome sequences and technologies have improved monitoring of the transcriptional reprogramming of cells due to environmental factors. However, further studies show that transcriptional factors are not perfect indicators of levels of protein in vivo fluxes (Griffin et al. 2002; Washburn et al. 2003; Daran-Lapujade et al. 2004), and thus it brings poor comprehension of complete biological systems. The use of sensitive and quick techniques to identify and technical improvement of protein by two-dimensional gel electrophoresis (2-DE) have made these techniques sophisticated for analysis. Proteomics is used for relatively fast, sensitive, and reproducible investigation of changes in the protein profile caused by introduction, mutations, or gene silencing due to various stress factors. This technique is important for generating information on physiological, biochemical, genetic, and architectural aspects of cells. Proteomics is an important technique for characterizing lines, individuals, and estimation of inter-/intrapopulation genetic variability, establishing genetic differences for use in phylogenetic studies, and characterizing the mutants with localized encoding of genes that indicated proteins (Thiellement et al. 1999). This is necessary for decoding the role and function of genes in plant genome sequencing projects. Use of proteomics can tremendously increase agricultural productivity (Dhand 2000; Xu et al. 2006). It is the most important method used for identification of proteins repressed, induced, or modified through posttranscription during the development as complex as senescence. Protein synthesis and accumulation in the seeds is dependent upon N supply from the mother plant during seed development. In legumes, sources of N could be (1) exogenous N absorbed from the soil N and/or N fixed by the symbiotic relationship with microbes and (2) from N mobilized from vegetative parts. Nitrogen fixed by microbes is not sufficient to fulfill the high N demand for seed development (Sinclair and de Wit 1976; Salon et al. 2001). Nitrogen mobilization for seed development closely related the shedding of vegetative parts which is due to the decrease in protein and chlorophyll and subsequently leaf yellowing.

Amount of available N and its source affected the complex and poorly understood metabolic rearrangement (ter Schure et al. 2000; Wek et al. 2004). Both limitations and availability of N had overrepresented the proteins in the category "metabolism" (42 proteins), which resulted in rearrangement of metabolic processes in yeast for adaptation under changed nutrient availability (Kolkman et al. 2006). Cánovas et al. (2004) characterized symbiotic proteins and defined the modifications in their metabolisms while interacting with each other. Proteome maps from control *Melilotus alba* roots, wild-type nodules, and cultured *Sinorhizobium meliloti* bacteroids were developed and compared. Sequencing of N present in amino acid and MALDI-TOF-MS peptide mass fingerprint analysis along with database searching were used for putative identification of about 100 nodule, bacterial, and bacteroid proteins. Deregulated proteins between the N-deficient media of C/N ratio and control have been identified to better understand the metabolism and regulation of N in the cultured *Monascus* cells under limited N supply (Lin et al. 2005). This study demonstrated that proteomics helps in the investigation of biological processes through systematic analysis of many expressed proteins that is a good approach to associate sequencing with functional genomics.

6.10 Conclusion

Nitrogen uptake, assimilation as well as utilization, is highly controlled by signalling pathways.

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7 Phosphorus Deficiency in Plants: Responses, Adaptive Mechanisms, and Signaling

Tariq Aziz, Muhammad Sabir, Muhammad Farooq, M. Aamer Maqsood, Hamaad Raza Ahmad, and Ejaz Ahmad Warraich

Abstract

Phosphorus (P) deficiency is a common nutritional factor limiting agricultural production around the globe. Application of phosphatic fertilizers is generally recommended to cope with P deficiency; however, low use efficiency of available P fertilizers both in calcareous and acid soils limits its viability and also had serious environmental concerns. Higher plants have adapted a number of mechanism to live with low available P in soil such as changes in root morphology and architecture, decreased growth rate, improved P uptake and utilization efficiency, and exudation of organic acids and enzymes to solubilize external inorganic and organic P compounds in the rhizosphere. Plant species and even cultivars widely differ in P efficiency because of differences in one or more of these mechanisms. Exploitation of these genetic variations among crop plants can sustain agricultural production. Understanding the mechanism involved in sensing P deficiency could facilitate selection, breeding, and genetic engineering approaches to improve crop production in P-stressed environments and could reduce dependence on nonrenewable inorganic P resources. In this chapter, we briefly reviewed the responses of P deficiency in higher plants, their adaptive mechanisms, and signaling pathways.

T. Aziz
Institute of Soil and Environmental Sciences, University of Agriculture, Faisalabad 38040, Pakistan

School of Plant Biology, University of Western Australia, Crawley, WA 6009, Australia
e-mail: draziz@uaf.edu.pk

M. Sabir (✉) • M.A. Maqsood • H.R. Ahmad
Institute of Soil and Environmental Sciences, University of Agriculture, Faisalabad 38040, Pakistan
e-mail: cmsuaf@gmail.com; mohamgill@uaf.edu.pk; khamaad@gmail.com

M. Farooq
Department of Agronomy, University of Agriculture, Faisalabad 38040, Pakistan

The UWA Institute of Agriculture, University of Western Australia, Crawley, WA 6009, Australia
e-mail: farooqcp@gmail.com

E.A. Warraich
Department of Crop Physiology, University of Agriculture, Faisalabad 38040, Pakistan
e-mail: uaf_ewarraich@yahoo.com

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Phosphorus • Phosphorus utilization efficiency • Phosphorus signaling deficiency • Organic acid efflux

7.1 Introduction

Phosphorus (P) is an essential macronutrient which constitutes about 0.2 % of plant's dry matter (Marschner 1995). Phosphorus is required during the process of energy generation and transfer, carbon metabolism, membrane synthesis, enzyme activation, and nitrogen fixation (Schachtman et al. 1998) and is a constituent of key biomolecules like nucleic acids, phospholipids, and adenosine triphosphate (ATP) (Marschner 1995). Limited P availability in soils is an important nutritional constraint to the growth of plant (Bates and Lynch 2000). Phosphorus is the least mobile nutrient under most soil conditions irrespective of total P contents in the soils (Hinsinger 2001; Schachtman et al. 1998). Soils can be classified into two major groups with respect to total P contents: soils containing inherently low-P contents like acrisols or sandy soils, and other group of the soils includes nitisols, acid andosols, or calcareous/alkaline soils that contain considerable amount of P, but major fraction is fixed with different soil constituents. In both type of soils, P concentration in soil solution is suboptimal and is generally in the range of 1–10 μM (Frossard et al. 2000; Mengel and Kirkby 1987; Ozanne 1980; Schachtman et al. 1998). Suboptimal P levels in soil solution can cause yield depressions up to 5–15 % of maximum crop yields (Shenoy and Kalagudi 2005). Application of P fertilizers is the most common practice to address the problem of low-P availability in agricultural soils (Ramaekers et al. 2010). However, this practice is confronted with daunting challenges of immobilization/precipitation of applied P with soil constituents, depletion of nonrenewable P sources, and high cost of P fertilizers (Vance et al. 2003). Available P in most of soils may constitute < 0.1 % of total soil P (Khan et al. 2009). In P-deficient soils, the use efficiency of applied P is very low and >80 % of applied P may be fixed on soil constituents or precipitation with Ca, Fe, and Al compounds and thus becomes unavailable to the plants (Gill et al. 1994; Trolove et al. 2003; Vance et al. 2003) or converted to organic forms (Holford 1997) and about 20 % or less of P applied is removed by the crop in the first year after its application. According to the US geological survey, globally, 22 million tonnes P extracts from natural sources annually (Gaxiola et al. 2011). Globally, P consumption is increasing about 3 % annually and natural reserves may be depleted in the near future (Cordell et al. 2009; Jasinski 2008).

Globally, the demand for P is increasing by 3–4 % (Maene 2007) and major demand is coming from Asian countries (Cordell et al. 2009). Modern agriculture is fully dependent on application of P fertilizers which are manufactured by using nonrenewable phosphate rock. The known reserves of phosphate rock are sufficient only of approximately 50–100 years (Smil 2000; Steen 1998). Strategies and management practices should be adopted to increase P use efficiency for sustaining agricultural production. This can be accomplished by breeding crops which are efficient in P acquisition or P use (Gill et al. 2004; Gill and Ahmad 2003), as presence of variability among different crop cultivars has been reported for P efficiency (El Bassam 1998; Kosar et al. 2002; Osborne and Rengel 2002; Ozturk et al. 2005; Singh Gahoonia and Nielsen 2004). An adaptation of cultivars which are efficient users of nutrients is an easy approach due to no additional costs and no major changes in cropping systems. Categorization of crop cultivars on the basis of growth and P uptake will be helpful in the identification of varieties which can be cultivated in different soils and selection of parents

for recombination breeding to develop P-efficient cultivars (Gill et al. 2004). Efficiency of applied P fertilizers can be increased by growing crop species/varieties efficient in P absorption and utilization and thus reduce the environmental degradation as well as input cost.

7.2 Adaptations of Plants to P-Deficient Environment

Plants have a wide range of adaptive mechanisms under P-deficient conditions to absorb sufficient P to maintain metabolic activities and growth (Lambers et al. 2010; Rengel and Marschner 2005). Physiological mechanisms relating to P-deficiency tolerance by crop plants have been reported by many of the earlier scientists (Krasilnikoff et al. 2003; Singh Gahoonia and Nielsen 2004), and some of these adaptations are listed in Table 7.1. Plant adaptations can be grouped into two major categories (Rengel and Marschner 2005; Vance et al. 2003), viz., acquisition efficiency and utilization efficiency. Acquisition efficiency is the capacity to absorb sparingly soluble nutrients like P, while utilization efficiency is the capacity to produce a large amount of biomass per unit of nutrient absorbed. Strategies which aim at reducing P use include decreased growth rate, enhanced growth per unit of P absorbed, remobilization of internal P, modifications in metabolism of carbon and alternative respiratory pathways (Uhde-Stone et al. 2003), and modifications in the biosynthesis of membrane requiring less P (Lambers et al. 2006; Plaxton and Carswell 1999; Uhde-Stone et al. 2003; Wasaki et al. 2003).

Exudation of phosphatases, release of organic acids from roots (Dakora and Phillips 2002; Gahoonia et al. 2000; Johnson and Loeppert 2006; Singh Gahoonia and Nielsen 2004; Vance et al. 2003), enhanced root growth with altered root architecture (Bucher 2006; Raghothama and Karthikeyan 2005; Singh Gahoonia and Nielsen 2004), root hair development, and enhanced expression of P_i transporters (Gilroy and Jones 2000) are responsible for enhanced P uptake.

Different plant species and genotypes within species differ in adaptive mechanisms for efficient P use. Intraspecific variations for P acquisition and utilization in several species are well documented (Aziz et al. 2006, 2011a; Gill et al. 2004; Lambers et al. 2010, 2011; Singh Gahoonia and Nielsen 2004). These variations may be attributed due to differences in relation to external critical levels of P; internal critical requirements; P uptake, transport, and utilization efficiencies; exudation pattern; root morphology; and expression of P_i transporter genes (Aziz et al. 2006; Singh Gahoonia and Nielsen 2004). Specific traits/mechanisms responsible for P efficiency in higher plants are briefly reviewed below.

7.2.1 Architectural Adaptations

Root architecture, defined as the spatial configuration of plant roots, is important for absorption of relatively less mobile nutrients like P (Lynch 2007; Zhu et al. 2005). Architectural adaptations are related to the change in root branching, root length, and formation of root hairs (López-Bucio et al. 2002; Richardson and Simpson 2011; Trachsel et al. 2011). Plants differ in the mechanisms for absorption of P from deficient environments. Efficient plant genotypes may have adaptations to explore more soil by increasing surface area, transforming plant-unavailable forms of nutrients in available forms, and take up nutrients across the plasma membrane (Rengel 2001). Plant roots perform a range of functions in plants like anchorage and absorption of nutrients and water (Bertin et al. 2003; Lambers et al. 2006, 2010; López-Bucio et al. 2003). Thus, the changes in the root architecture could affect nutrient and water absorption by plants. Root architecture is affected by three major processes, viz., (1) cell division at the primary root meristem which determines growth by adding new cells to the root, (2) lateral root formation which improves exploration of soil by the roots, and (3) development of root hairs, thereby increasing the root surface area (López-Bucio et al. 2003). The rooting pattern of plants is mainly determined by

Table 7.1 Plant adaptive mechanisms to cope with P deficiency in soil

Trait	Efficient genotypes	Inefficient genotypes	References
P contents in harvested portion	Low	High	Marshall and Wardlaw (1973) and Richardson and Simpson (2011)
Internal critical P concentration	Low	High	Hammond et al. (2009), Lambers et al. (2011), and Ozturk et al. (2005)
Number of adventitious roots	More	Less	Bates and Lynch (2001), Gahoonia et al. (1999), Jungk (2001), Liao et al. (2001), Lynch and Brown (2008), Richardson and Simpson (2011), Trachsel et al. (2011), and Walk et al. (2006)
Root diameter	Fine	Course	
Root volume	High	Low	
Rooting density	High	Low	
Root architecture and root growth angle	More shallower roots exploring surface soil	Less shallower roots	
Root hairs	More and long	Less and short	
Root exudates	Higher amounts	Lower amounts	Lambers et al. (2010, 2011), Aziz et al. (2011a), Pearse et al. (2006a), Pang et al. (2010), Richardson and Simpson (2011), Gregorge et al. (2008), Ma et al. (2009)
Organic acids/anions			
Phosphatases			
Internal P utilization	High	Lower	Lambers et al. (2010), Aziz et al. (2006, 2011a), Hammond et al. (2009), and Ozturk et al. (2005)
P remobilization	Yes	No or minimum	Aziz et al. (2011b), Lambers et al. (2010), Akhtar et al. (2008), Nagarajan et al. (2011), Lovelock et al. (2006)
Specialized root structures	Cluster roots	No or very little cluster roots	Shane and Hans (2005), Pearse et al. (2007), and Lynch (2007)

the plant species, soil structure, and their interaction (Bertin et al. 2003). The larger root system provides more surface area for nutrient absorption by increasing the contact with soil which plays an important role for P absorption (Aziz et al. 2011a; Gahoonia and Nielsen 1998; Lynch 2007; Römer and Schenk 1998).

Distribution of nutrients in soils is heterogeneous or patchy. In order to enhance nutrient uptake, plant roots have to exploit these nutrient patches. Asymmetrical development and exceptional flexibility in architectural patterns of roots enable plants to exploit soils and allow root proliferation in nutrient-rich zones (Lynch 1995). Depending upon the plant species and environmental conditions, the root systems vary widely in terms of root architecture, including number and size of root hairs (Lambers and Shane 2007; Lynch and Brown 2001; Shane et al. 2006).

Various root characteristics including root architecture, root diameter, root hairs, cluster roots (Bates and Lynch 2000; Hill et al. 2006; Singh Gahoonia and Nielsen 2004), symbiotic relationship with mycorrhiza, kinetics of P uptake, and rhizospheric processes (Aziz et al. 2011a; Hinsinger 2001; Pang et al. 2010; Ryan et al. 2009) cause variations in P uptake among the plant species/cultivars (Lynch and Brown 2001; Singh Gahoonia and Nielsen 2004). Response to P deficiency by plant roots involves changes in root architecture and the shift of biomass allocation from basal to adventitious roots in such a way to explore more topsoil or P-rich (Lambers and Shane 2007) sites for P acquisition (Liao et al. 2001, 2004; Lynch and Brown 2001). These adaptations include horizontal basal root growth, increased adventitious root formation, enhanced lateral root formation, and increased root hair density and length (Bonser et al. 1996; Liao et al. 2001; Lynch 2007). Topsoil foraging is strongly associated with P acquisition in low-P soils (Rubio et al. 2003; Zhu et al. 2005) due to

low mobility of P in soil. Low P in the rooting zone favors the formation of lateral roots (López-Bucio et al. 2002; Lynch 2007). A large root surface area is achieved by a combination of reduced mean root diameter and elongation of relatively thinner roots (Fitter et al. 2002). Root diameter is very important in exploration of soil volume by roots as it determines the volume of soil that can be explored by the roots (Fitter 1991; Gahoonia et al. 2006). Plants with a smaller root diameter can explore more soil per unit of root surface area (Fitter et al. 1991) and can efficiently uptake P from limiting environments (Singh Gahoonia and Nielsen 2004).

Root hair enhances the ability of roots to explore the rhizosphere for P due to increased surface area for absorption (Hill et al. 2010; Ma et al. 2001b; Zhu et al. 2010). Root hairs constitute up to 77 % of the total root surface area and thus are the major point of contact between plants and the rhizosphere (Föhse et al. 1991; Gahoonia and Nielsen 1998). Under P deficiency, increased root hair density and length is well documented in legumes (Yan et al. 2004) and barley (Gahoonia and Nielsen 1998). Ma et al. (2001a) reported that root hair density in *Arabidopsis thaliana* was high under P deficiency. Oilseed rape was reported to contain large amount of P as compared to maize despite having less root and shoot biomass (Morel and Hinsinger 1999), and this could be due to long root hairs of oilseed rape compared to maize (Gahoonia and Nielsen 2004). Large differences in root morphology and distribution are present between genotypes of many plants (Bates and Lynch 2001; Krasilnikoff et al. 2003; Römer and Schenk 1998; Vance 2001). Root characteristics such as total root length, root hair length and density, and specific root length have been shown to vary considerably between genotypes of several species (Løes and Gahoonia 2004; Nielsen et al. 1997; Römer and Schenk 1998; Singh Gahoonia et al. 1997; Yan et al. 1995). Significant differences in P uptake in cereal cultivars grown on low-P soil were reported due to differences in length of root hairs of these cultivars (Singh Gahoonia et al. 1997). Similarly, genetic differences in P uptake in cowpea and in maize due to variation in root length and root hairs have been reported (Krasilnikoff et al. 2003).

Maize genotypes modified their root architecture in response to low P in the rooting medium (Zhu et al. 2005). Genotypes which performed better under P-deficient conditions develop shallow root systems to tap P accumulated in topsoil and have greater specific P absorption rate, tissue P contents, relative growth rate, and biomass accumulation than others (Zhu et al. 2005). Phosphorus availability regulates different aspects of root architecture like axial extension, root branching, basal root gravitropic, the relative distribution of basal root length, and adventitious roots (Liao et al. 2004; Miller et al. 2003; Ochoa et al. 2006).

These differences raise the possibility of selection and breeding of crop genotypes having extensive root systems to cope with P deficiency in soils. Earlier studies showed that root growth (root size, root weight, etc.) positively correlated with biomass production in different crops (Barraclough 1984; Gill and Ahmad 2003; Kosar et al. 2002; Olaleye et al. 2011).

7.2.2 Root Biomass

Preferred biomass partitioning towards the roots is one of the most important adaptive mechanisms of the plants under P-deficient conditions (Hermans et al. 2006; Mollier and Pellerin 1999). Plants allocate more assimilates towards those areas which are directly involved in nutrient acquisition (Marschner 1995). Difference in preferred biomass partitioning between roots and shoots of the plants grown under P-deficient and sufficient supplies could be ontogenetic (comparing the plants at different growth stages) rather than a truly plastic response (Kemp and Blair 1994; Niklas 1994). However, it is evident that P supply influences biomass partitioning directly independent of ontogeny (de Groot and Grubmüller 2001; Ryser et al. 1997). Mollier and Pellerin (1999) reported that root:shoot ratio of maize significantly increased in P-deficient plants as compared to those grown with sufficient P

supply, and this could be due to preferential distribution of carbohydrates towards roots under P-deficient conditions. Phosphorus deficiency causes accumulation of carbohydrates in roots and thus increases the root:shoot ratio of the plants (Cakmak et al. 1994; Hermans et al. 2006).

7.2.3 Phosphorus Uptake

Phosphorus moves to the plant roots by diffusion due to its strong reactions with soil constituents (Hinsinger et al. 2006; Rahmatullah et al. 1994; Trolove et al. 2003). Plants absorb P ions actively across the plasmalemma against the concentration gradient between soil solution and roots (Schachtman et al. 1998) as concentration of orthophosphates is usually very low (Bieleski 1973; Schachtman et al. 1998).

Plant species and cultivars differ greatly in their growth responses to low-P supply in the rooting medium (Gill et al. 2004; Gill and Ahmad 2003; Veneklaas et al. 2012). Some plants can grow efficiently in low-P soils mainly because they can take enough P for their optimum growth. Genotypes that are more efficient in P acquisition from deficient conditions are generally considered better adaptable to P deficiency in soils and their impact on adaptations to P stress (Duncan and Baligar 1990; Liu et al. 2004; Osborne and Rengel 2002; Rengel and Marschner 2005). Variations in nutrient acquisition from the rooting medium are attributed to variations in root plasticity in response to nutrient status, differences in uptake along the roots, and plant growth rate (Gahoonia and Nielsen 2004; Krasilnikoff et al. 2003). These may include morphological features as well as the biochemical mechanisms responsible for the initial transfer of ions across root cell membranes (Bates and Lynch 2000; Schachtman et al. 1998; Vance et al. 2003).

Naturally, plants can accumulate P and other nutrients selectively and actively in their tissues (Marschner 1995). Uptake kinetics explains the relationship between concentration of nutrient in soil solution and its influx into the root, and it can be explained in terms of a modified Michaelis-Menten kinetics (Nielsen 1972). Later on, idea of dual phasic P transport system which operates at low or high nutrient concentration in external solution was proposed (Epstein and Leggett 1954), and uptake systems were classified as low- and high-affinity uptake systems. Two independent Michaelis-Menten-type systems have been proposed in the literature under varying levels of P supply. Ullrich-Eberius et al. (1984) reported two P uptake systems with significantly different K_m values. The value of K_m in both systems was higher under P depletion. High-affinity system is inducible and operates when external P concentration is very low (Clarkson and Scattergood 1982; Smith et al. 2000). As P concentration in the rhizosphere is very low (usually in μM range), only high-affinity uptake system operates in soil (Raghothama 1999).

Buhse (1992) proposed that differences in influx at the same concentration in solution are related to the uptake kinetics parameters, I_{max} (maximum uptake rate), K_m (external concentration at which uptake rate is half of I_{max}), and C_{min} (minimum concentration at which influx ceases). Several authors reported that large reductions in shoot P concentration were related to an increase in I_{max} (Anghinoni and Barber 1980; Drew 1984). Jungk and Barber (1974) proposed that increased I_{max} under low shoot P concentration may be due to new root development during starvation period or increased concentration gradient with root P contents. I_{max} value should be adjusted according to P concentration in plant and possibly root radius (Anghinoni and Barber 1980). The C_{min} value indicates the lowest external concentration below which plants are unable to take up P from solution. Plants able to take up P at very low concentration in the soil solution (C_{min}) would be more efficient in P acquisition. Differences in values of uptake kinetics among several crop cultivars are well reported (Asher and Loneragan 1967; Nielsen and Barber 1978; Nielsen and Schjørring 1983).

Molecular research has revealed that plants have both a low- and high-affinity P uptake system (Bieleski 1973; Muchhal and Raghothama 1999). High-affinity systems are induced at low-P conditions (Furihata et al. 1992), while low-affinity system appears to be constitutive in

plants (Raghothama 1999). Multiple plasmalemma P transporters differentially express under varying P nutritional regimes (Epstein and Leggett 1954; Plaxton and Carswell 1999). The high-affinity transporter mRNA transcripts in roots increased under low external P concentration for increased capacity of roots for P uptake (Duncan and Carrow 1999; Shenoy and Kalagudi 2005). These high-affinity transporters play an important role in the P acquisition under P-deficient root environment.

Bhadoria et al. (2004) reported the existence of differences in P use efficiency in maize and groundnut grown in solution culture which were related to differences in uptake kinetics of the two species. These differences observed in solution culture were opposite to those observed in the field (Bhadoria et al. 2004) and were probably based on several other edaphic factors other than uptake kinetics. Gahoonia and Nielsen (2004) proposed that genotypes should be selected for high I_{max} values and root length and low for C_{min} and K_m values. Lower C_{min} values show the ability of plants to uptake P at low concentration in soil solution that could be important in future low-input sustainable agriculture systems particularly in developing countries. They further pointed out that the importance of uptake kinetics parameters of plants in the soil plant system is still to be investigated. As P is moved in soil through diffusion, uptake efficiency of plants is of minor importance in the P acquisition (Barber 1995) than different P acquisition traits such as root dry matter, root length, no. of root hairs, and root hair density. Barber and Mackay (1986) and Krannitz et al. (1991) also reported that differences among cultivars of a species in P uptake can be explained by differences in root growth.

7.2.4 Nutrient Translocation/ Remobilization Within the Plant

Movement of nutrients within the plant body under deficient conditions is another mechanism adopted by plants to cope with deficiency. Salinas and Sánchez (1976) proposed that these differences in nutrient movements under varying levels of nutrient supply are adjustments to adapt to deficiency. The capability of plants for retranslocation of P from one plant part to another is an adaptive strategy used by some plants under low-P supply (Gill and Ahmad 2003).

Efficient redistribution and reutilization of nutrients from deficient or senescent plant parts could also cause variations in nutrient utilization. Adu-Gyamfi et al. (1989) observed increased rate of absorption and translocation of P to leaves under P deficiency. Several researchers have reported that relatively lower proportions of total P was retained in roots and stems and higher proportions were translocated to leaves under P deficiency in P-efficient cultivars than inefficient cultivars (Snapp and Lynch 1996).

In P-deficient plants, limited P supply to the shoots from the roots via the xylem is supported by enhanced remobilization and retranslocation of stored P in mature leaves to the younger leaves. This compartmentation of P in various plant organs is reported to be inducible under conditions of P deficiency (Gerloff and Gabelman 1983) and is under genetic influence (Schachtman et al. 1998). Internal P concentration at a critical location in plants plays a regulatory force for enhanced P uptake from the root medium under P deficiency (Drew and Saker 1984; Lefebvre and Glass 1982).

7.2.5 Phosphorus Utilization Efficiency

Nutrient utilization is defined as the amount of biomass produced per unit amount of nutrient absorbed (Fageria and Baligar 1997; Siddiqi and Glass 1981). It has been used to compare the efficiencies of nutrient utilization among various cultivars or species. PUE is the ability of crop cultivars to grow well under low available P concentrations. Plants efficient in nutrient utilization may enhance the efficiency of applied P.

Efficient P use within the cell is another adaptive strategy in many crop species. Nanamori et al.

(2004) reported significant differences in P use efficiency among forage grass and rice. Variations for P use efficiency among cultivars of wheat (Batten 1993; Kosar et al. 2002), barley (Römer and Schenk 1998), maize (Elliott and Læuchli 1985), rice (Aziz et al. 2006; Gill and Ahmad 2003), forage crops (Mugwira and Haque 1993), and several other crop species (Föhse et al. 1991).

Some physiological and metabolic P transformations may also take place in plants as a response to P deficiency including induction of phosphate scavenging and recycling enzymes, organic acid excretion (Duff et al. 1994), induction of metabolic phosphate recycling enzymes (Plaxton and Carswell 1999), alternative pathways of cytosolic glycolysis (Plaxton and Carswell 1999), increased tonoplast H^+-,pumping pyrophosphatase, and alternative pathways of electron transport (Gonzàlez-Meler et al. 2004).

Phosphorus use efficiency is of special interest to developing countries, especially having soils low in available P. Tailoring plants to adapt to conditions of low-P supply and yield more from each unit of applied P is considered an alternative for high-input agriculture. An overall high PUE of plants can be achieved through the combined effect of P uptake efficiency and P utilization efficiency (Römer and Schenk 1998). As both these traits are genetically heritable (Nielsen and Schjørring 1983), they should be included in genetic improvement programs through breeding (Gill et al. 2004). An adaptation of nutrient-efficient crop cultivars is relatively easy, since no additional costs are involved and no major changes in cropping systems are necessary. Currently breeders are working on the selection or development of responsive cultivars, but traits responsible for differences in PUE should be considered in breeding programs such as P acquisition, translocation, and internal utilization and would be considered in breeding programs for low-input sustainable agriculture systems (Gill et al. 2004; Ortiz-Monasterio et al. 2007).

Cultivars can be categorized based on P use efficiency as discussed by Aziz et al. (2006), Fageria and Baligar (1997, 1999), and Kosar et al. (2002) into four groups as (1) efficient and responsive, (2) efficient and nonresponsive, (3) inefficient but responsive, and (4) inefficient and nonresponsive (Kosar et al. 2002). Fageria and Baligar (1999) compared different growth and P uptake parameters of efficient and responsive cultivars with inefficient and nonresponsive cultivars. They concluded that greater P efficiency in wheat genotypes was due to P use efficiency rather than to differences in P concentration. Gardiner and Christensen (1990) also reported that greater P efficiency in wheat genotypes was due to greater use efficiency rather than variations in P uptake. Recently Gill et al. (2004) studied P use efficiency of 30 wheat genotypes. They reported significant differences among the genotypes for biomass, P efficiency, harvest index, and grain yield. They categorized wheat genotypes into nine groups by regressing dry matter yield (*X*-axis) and P uptake (*Y*-axis).

7.2.6 Organic Acid Efflux

Plant roots exude a variety of carbon (C) compounds (simple sugars, organic acids, amino acids, phenolics, enzymes, other proteins) and inorganic ions (protons, phosphate, other nutrients, etc.) into the rhizosphere (Crowley and Rengel 1999; Jones and Darrah 1995; Marschner 1995; Miller et al. 2001; Rengel 2001; Veneklaas et al. 2003). Root exudation influences significantly rhizosphere chemistry, soil microflora and fauna, and plant growth (Hinsinger 2001; Johnson et al. 1996; Mench et al. 1987; Vance et al. 2003) and is involved in nutrient acquisition (Neumann et al. 2000; Römheld and Marschner 1990). Root exudates are a major source of energy for microbial growth in soils (Bowen and Rovira 1999; Rengel and Marschner 2005; Uren and Reisenauer 1988).

Nature of root exudates varies significantly in response to environmental stress especially under nutrient deficiency, e.g., P, Fe, Zn, and Mn (Jones and Brassington 1998; Jones and Darrah 1995; Neumann and Martinoia 2002). Among these diverse C compounds, organic acids and sugars are important for the mineral nutrition of plants as well as for microbial growth in the rhizosphere (Jones and Darrah 1995; Mench et al. 1987).

Under P-deficient conditions, exudation of organic acids like acetic, aconitic, citric, malic, fumaric, lactic, oxalic, and succinic acids increases in many plant species (Grierson 1992). Organic acids help in increasing the availability of P and micronutrients because the organic anions can compete with Pi for complexation by Fe, Al, and Ca (Gerke et al. 2000; Hinsinger 2001; Römheld and Marschner 1990) and may hydrolyze organic P (Gerke et al. 2000; Gerke and Meyer 1995). Increased exudation of organic acids under P deficiency has consistently been reported in many plant species such as in white lupin (Johnson et al. 1996; Neumann et al. 2000; Neumann and Römheld 1999), alfalfa (Lipton et al. 1987), and oilseed rape (Hoffland et al. 1989, 1992).

Kihara et al. (2003) reported significant increase in citrate release by rice under P deficiency. Citric acid can enhance significantly mobilization of P from Ca compounds by reducing the pH (Dinkelaker and Marschner 1992). Increased secretions of malic and citric acid in root zone of *Brassica napus* L (Aziz et al. 2011a, b; Hoffland et al. 1989) P deficiency was highly effective in increasing P uptake from sparingly soluble rock P. On the other hand, Wouterlood et al. (2005) reported that carboxylate exudation in chickpea did not correlate with P availability. However, di- and tricarboxylic acids have the ability to increase P solubility also in P-fixing soils and hence strongly improve P acquisition of plants grown in soils low in available P.

Species and genotypes which are tolerant to P deficiency differ in exudation of root secretions responsible for P solubilization. Cieśliński et al. (1998), Neumann and Römheld (1999), and Pearse et al. (2006b) reported significant differences among 13 plant species for amount of organic anion release under P deficiency. The amounting of the exudates also varied significantly between species.

The activity of phytase and acid phosphatase increased in root exudates in various species and genotypes within species grown under low-P supply (Asmar 1997; Lambers et al. 2010; Li et al. 1997; Pearse et al. 2006a; White and Veneklaas 2012). The P-deficiency-tolerant genotypes of different crops had a greater activity of extracellular phosphatases in the rhizosphere soil than genotypes sensitive to P deficiency (Asmar et al. 1995; Gerke et al. 2000; Richardson and Simpson 2011).

7.3 Gene Expression Under Phosphorus Deficiency

Response to phosphate deficiency involves a set of morphological, biochemical, and physiological changes in metabolic expression, which enables the plants to adapt to P-limited environments. Manipulation of the gene expression under P-deficient conditions could improve the PUE of plants.

A series of genes is involved in the adaptations, through the regulation of P acquisition, internal remobilization, change in metabolism, and signal transduction to P deficiency (Fang et al. 2009). For example, the expression of genes encoding ribonucleases (RNS) and purple acid phosphatases (PAPs) is generally upregulated in plants raised in P-limited environments. Ribonucleases help the plants to release P from organic sources and make that plant available; RNS also help in mobilization of the organic P in soil for plant uptake (Bariola et al. 1994; Duff et al. 1994). Likewise, genes responsible for phosphate transporters (PTs) are the major P-deficiency-induced genes which are isolated and characterized from different plant species (Liu et al. 2001; Miller et al. 2001). Several split-root experiments indicated that P-deficiency-induced genes are regulated by P status of the plant and not the soil P concentration (Burleigh and Harrison 1999; Liu et al. 1998; Shane et al. 2003).

Phosphorus uptake1 (*Pup1*), a major quantitative trait locus (QTL), involved in tolerance of soil P deficiency is located on rice chromosome 12 (Shane et al. 2003). As highly branched root systems with long root hairs are helpful in improving PUE (Ramaekers et al. 2010), Pup1 stimulates the root growth (Gamuyao et al. 2012; Li et al. 2008), increases the P uptake (Wissuwa and Ae 2001), and increases grain yield substantially (Chin et al. 2010). Similarly, white lupin

exhibits morphological and gene expression changes in P-deficient roots. Phosphate transporter (*LaPT1*) and secreted acid phosphatase (*LaSAP1*) promoter-reporter genes isolated from lupin showed significant induction in roots specifically in response to P deficiency when transformed into alfalfa.

The identification of transcriptive factors, regulatory signaling cascades, and genes associated with plant responses to P stress could be helpful for developing crops with improved P use efficiency. However, since several studies have assayed gene expression in plants under controlled environment, the outcomes of such experiments need to be confirmed under field conditions.

7.4 Signaling and P Deficiency

Plant growth and development is determined by environmental factors like temperature, light intensity, water, and essential nutrients. Plants allocate new biomass to the organs used for acquiring the scarce resources to adjust the ionic imbalance (Marschner 1995). Many studies have focused on the relationships between nutrition, plant growth, and development and have explained the mechanisms of ion transport and the biochemical pathways influenced by mineral scarcity (Hermans et al. 2006; Chandna et al. 2012; Hakeem et al. 2012a, b). Although understanding about the molecular and physiological processes involved in sensing, signaling, and allocation of deficient nutrients is poor, but with onset of microarray technologies to study gene expression, study of transcriptional changes associated with mineral imbalance has become easier (Hammond et al. 2004). Deficiency of P results in preferential allocation of carbohydrates towards the roots and thus increases root to shoot ratio. This also results in modification of photosynthesis, metabolism of sugar, and/or partitioning of carbohydrate between source and sink tissues (Sánchez-Calderón et al. 2006). The knowledge about how the plants sense P deficiency is growing rapidly, but a lot is still to be discovered in the near future. Phosphorus deficiency in plants triggers many transcriptional, biochemical, and physiological changes that ultimately help the plants absorb P from the soil or improve the P use efficiency (Amtmann et al. 2005; Hammond and White 2008). Plants have evolved various adaptive mechanisms to absorb P under P-stressed conditions which involve diverse developmental and biochemical processes. Different transcription factors that control response of plants under P starvation has been identified. The miRNAs are responsible for Pi homeostasis and signaling through the identification and characterization of PSR miRNAs under P-deficient conditions (Kuo and Chiou 2011). Understanding the mechanism involved in sensing P deficiency could facilitate selection, breeding, and genetic engineering approaches to improve crop production in P-stressed environments and could reduce dependence on nonrenewable inorganic P resources.

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8 Reactive Oxygen Species-Associated Mechanism of Acclamatory Stress Tolerance, Signaling and Redox-Regulated Gene Expression in Plants

Soumen Bhattacharjee

Abstract

The conflict between the role of reactive oxygen species (ROS) as toxic component and as a central player of signaling network is largely unresolved. It is becoming evident that ROS and oxidative burst during any kind of stress are recognized by plant as signal molecule for triggering defense response. The molecular mechanism associated with ROS-mediated signal transduction, leading to changes in gene expression, is one of the early responses in the acclamatory performance of plant. In *Arabidopsis*, a network of several genes is involved in managing the titer of ROS. This network of ROS-sensitive genes is highly dynamic and redundant that encodes redox-sensitive proteins and ROS-scavenging and ROS-producing molecules. The ROS-induced redox cues and associated retrograde signaling are extremely important in maintaining normal energy and metabolic fluxes, optimizing different metabolic and cellular functions, thereby controlling different acclamatory responses and ultimately the whole plant systemic signaling cascades. An overview of literature is presented on signaling role of ROS in plant acclamatory defense responses, along with their dynamics and specificity. In this context an effort has been made to update the concept of ROS signaling while addressing the significance of stress acclamatory performance of plants.

Keywords

Reactive oxygen species • Oxidative stress • Redox regulation • Stress acclimation • Transcriptome • Redox-regulated gene expression

8.1 Introduction

The present research concern in agriculture pertaining to plant responses to environmental stress is becoming increasingly significant in the perspective of rapid changes in climatic condition.

S. Bhattacharjee (✉)
Department of Botany, Krishnagar Government College (West Bengal Education Service), Krishnagar, Nadia 741101, West Bengal, India
e-mail: soumen1995@yahoo.com

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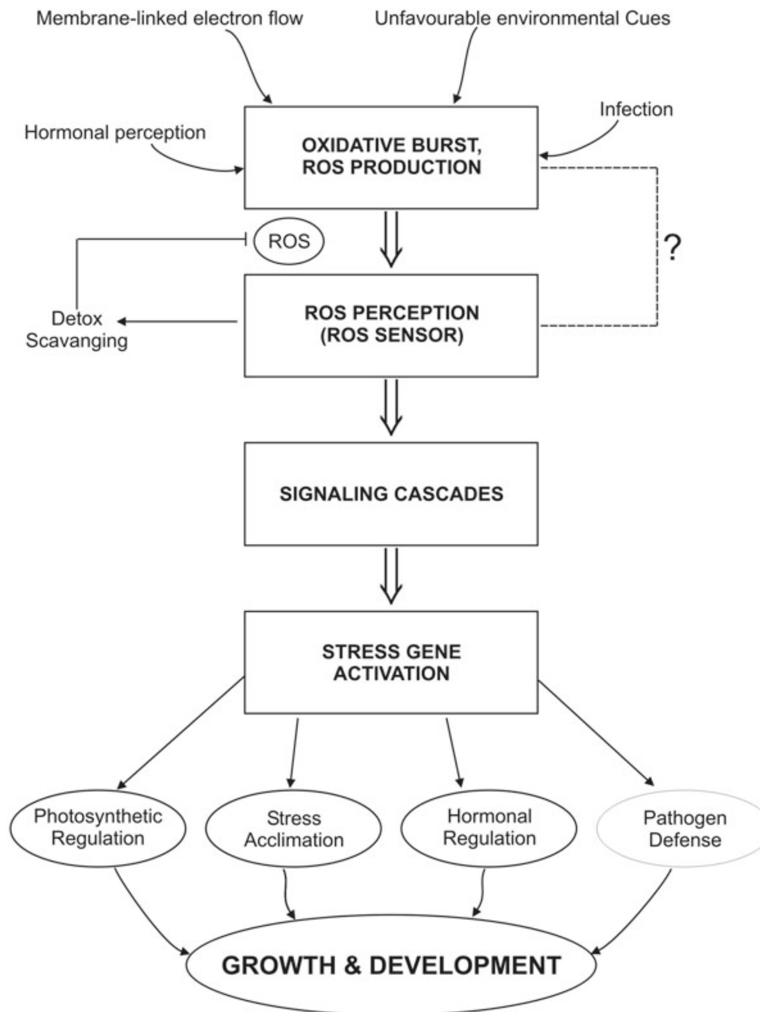


Fig. 8.1 Gross ROS network showing flow of information (production and perception of ROS, signaling cascades, gene activation followed by regulation of growth development). Different environmental and cellular signals result in enhanced generation of ROS or oxidative burst in cells by ROS-producing pathways of the network. Subsequently, ROS perceived by different ROS sensors and activate downstream signaling cascades. The intensity, duration, and localization of ROS signals are determined by interplay between ROS-producing and detox-scavenging pathways. Modulation of ROS level might also involve positive feedback loop between ROS perception and ROS production. In the downstream signaling cascades, stress inducible genes are activated that ultimately regulates growth and development by controlling photosynthesis, hormonal physiology, and pathogen defense and stress acclimation

Drought in combination with extremes of temperature and excess photochemical energy has been regarded as the most important environmental constraints for plant survival and productivity (Boyer 1982; Grover et al. 2009). Therefore, understanding the mechanism of abiotic stress tolerance is of utmost significance from the point of view of crop improvement and productivity.

Plant responses to unfavorable environmental cues primarily involve perception mechanism followed by signal transduction cascades and ultimately the differential expression of large array of stress inducible genes (Fig. 8.1). Identifying the signaling cascades and regulatory conditions that lead to differential expression of genes under environmental stress is therefore the central area of

Plant Stress Biology. Apart from the transcription factors, certain common regulatory signaling components in the form of reactive oxygen species (ROS) found to play as central player in controlling the signaling cascades and stress-related gene activation mechanisms (Fig. 8.1).

Plants are constantly subjected to changes in environmental cues. Being poikilothermic and static, they need to alter their metabolism in order to maintain cellular homeostasis mainly for balancing energy generation and consumption. The cellular homeostasis, particularly under stress, largely depends on a delicate signaling network that coordinates vital life processes like photosynthesis, photorespiration, and dark respiration. These processes in turn largely depend on membrane-linked electron transfer and the production of reductants and metabolic energy (Foyer and Noctor 2009; Suzuki et al. 2011; Bhattacharjee 2012b). An inevitable result of membrane-linked electron transport is the spilling of electrons onto molecular oxygen in plant cells, with the production of partially reduced toxic reactive oxygen species or ROS (Asada 1994; Varnová et al. 2002; Arora et al. 2002; Bhattacharjee 2005, 2012b; Suzuki et al. 2011). The imposition of any kind of stress further disturbs the redox homeostasis of the cell, resulting in the accumulation of ROS (Arora et al. 2002; Bhattacharjee 2005; Alscher and Hess 1993; Suzuki et al. 2011; Varnova et al. 2000). It has been estimated that 1 % of O_2 consumed by plants is diverted to produce ROS in various subcellular loci (Asada and Takahashi 1987; Eltsner 1987; Foyer and Noctor 2009). ROS also plays critical role during natural course of senescence, as evidenced by the higher level of ROS, and associated oxidative damages to thylakoid and inner mitochondrial membranes (Thompson et al. 1987; Vacca et al. 2004).

In fact, any condition in which cellular redox homeostasis of the cell is disrupted that is manifested in the form of an imbalance in which the redox steady state of the cell is altered in the direction of prooxidants can be defined as oxidative stress. The ROS capable of inducing oxidative stress and causing oxidative damage include superoxide ($O_2^{\bullet -}$), perhydroxyl radical ($HO_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$), alkoxy radical ($RO^{\bullet}$), peroxy radical ($ROO^{\bullet}$), singlet oxygen ($^1O_2$), and organic hydroperoxide (ROOH) (Table 8.1). The antioxidative defense system comprising of various scavenging enzymes and quenchers encoded by ROS network can be found in almost every subcellular compartment of plant cell (Table 8.1). Usually more than one enzymatic scavenging system against a particular ROS exists in almost every important compartment of the cell. It not only offers an efficient process of ROS detoxification but also enables the system to have the flexibility to tightly control the titer of ROS (Mittler et al. 2011; Bhattacharjee 2012b). However, the modes of coordination between different components of ROS removal network of plants, particularly under environmental stress, are extremely complicated (Karpinski et al. 1997; Mittler et al. 2011) and still remain largely unexplained.

Plant metabolisms are extremely sensitive to changing environmental cues. In most of the cases, metabolic imbalances caused by environmental stresses induce a secondary oxidative stress, causing oxidation of cellular components, affecting organellar or cellular integrity, and ultimately affecting growth and development. Some oxidized metabolites, including the products of lipid peroxidation, carbonylated proteins, which are traditionally being regarded as markers of oxidative stress, are now suggested to function as signaling intermediates under oxidative conditions (Spiteller 2003). Therefore, a close association between the redox states of different organelles can largely affect energy balance and carbon metabolism in plant cells. A high degree of metabolic coordination is required to maintain energy flow through chloroplast and mitochondria under environmental stress to avoid loss of redox homeostasis due to excessive generation of ROS (Suzuki et al. 2011; Takahashi and Murata 2008). In order to achieve this, a tight metabolic coordination and control is required for redox regulation of proteins, activation of sensor-mediated pathways, and activation of signaling pathways by ROS-responsive regulatory genes (Suzuki et al. 2011; Bhattacharjee 2012b).

Table 8.1 The common components of ROS and the antioxidative defense system that operate in plant cell. [A] The important ROS in plant tissues and their basic properties (Half-life, in biological system; migration capacity, distance traveled in one half-life time if the diffusion coefficient is assumed to be 10^{-9} m^{-2} s^{-1}). [B] Antioxidant mechanisms that modulate the level of ROS (through ROS removal mechanisms) in plant

[A]

ROS	Half-life	Migration capacity	Cellular location	Interacts with			References
				DNA	Protein	Lipid, carbohydrate	
Superoxide (O_2^-)	1–4 μs	30 nm	Membranes, chloroplast (Mehler reaction), mitochondria	No	Yes (Fe center)	Hardly	Halliwell and Gutteridge (1989), Bhattacharjee (2012b), Miller et al. (2010), and Fridovich (1995)
Hydrogen peroxide (H_2O_2)	1 ms	1 μm	Membranes, chloroplast, mitochondria, peroxisome	No	Yes (cysteine)	Hardly	Halliwell and Gutteridge (1989), Bhattacharjee (2012b), and Foyer et al. (1997)
Hydroxlyl radical ($OH^•$)	1 μs	1 nm	Chloroplast, membranes, mitochondria	Rapidly	Rapidly	Rapidly	Halliwell and Gutteridge (1989) and Bhattacharjee (2012b)
Singlet oxygen ($^1O_2$)	1–4 μs	30 nm	Chloroplast, membranes, mitochondria	Yes (guanine)	Trp, His, Tyr, Met, Cys.	PUFA	Halliwell and Gutteridge (1989), Bhattacharjee (2012b), and Foyer et al. (1997)
Alkoxy radicals ($RO^·$)	?	1 nm	Membrane lipid peroxidation	No	Yes	PUFA	Halliwell and Gutteridge (1989), Bhattacharjee (2012b), and Foyer et al. (1997)
Peroxy radicals ($ROO^·$)	?	1 nm	Membrane lipid peroxidation	No	Yes	PUFA	Halliwell and Gutteridge (1989) and Foyer et al. (1997)

[B]

Antioxidant system	Interacts/removes (product)	Cellular location	References
Superoxide dismutase	$O_2^{•-}$ (H_2O_2)	Chl, Cyt, Mit, Per	Halliwell and Gutteridge (1989), Bhattacharjee (2012b), Foyer et al. (1997), and Imlay (2008)

Catalase	H_2O_2 (H_2O)	Mit?, Per	Foyer et al. (1997) and Winston (1990)
Peroxidases	H_2O_2 (H_2O)	Many locations	Imlay (2008) and Eltsner (1987)
Ascorbate/glutathione cycle	H_2O_2 (H_2O)	Chl, Cyt?, Mit, Per	Alscher and Hess (1993)
Glutathione S-transferase	$ROO^{\cdot}$	Nuel, Cyt	Alscher and Hess (1993)
Halliwell –Asada Path way	$O_2^{\cdot-}$ (H_2O_2), H_2O_2 (H_2O)	Chl	Halliwell and Gutteridge (1989) and Foyer et al. (1997)
Glutathione peroxidases	H_2O_2 (H_2O)	Chl, Cyt, ER, Mit	Creissen et al. (1999)
	Lipid hydroperoxides		
	Other hydroperoxides		
Peroxiredoxin system	H_2O_2 (H_2O)	Chl, Cyt, Mit, Nuel	Foyer (1996) and Rouhier et al. (2009)
	Alkyl hydroperoxides		
	Peroxynitrite		
Thioredoxin system (regulating SH/S = S ratio)	H_2O_2 (H_2O)	Chl, Cyt, Mit	Rouhier et al. (2009)
Glutaredoxin system (regulating SH/S = S ratio)	H_2O_2 (H_2O)	Chl, Cyt, Mit, Sec	Foyer and Noctor (2009)
	Hydroperoxides		
Carotenes and tocopherol	$^1O_2$ (O_2)	Chl	Davison et al. (2002)

Chl chloroplasts, *Cyt* cytosol, *ER*, *Mit* mitochondria, *Nuel* nucleus, *Per* peroxisomes, *Sec* secretory pathway, *SH/S = S* sulfhydryl/disulfide ratio

Several advantages are associated with ROS functioning in plants. The capacity of cell to rapidly produce and scavenge them simultaneously under environmental stress enables plant with a system capable of rapid and dynamic changes in ROS titer and their subsequent spatiotemporal control. The selective advantage of ROS functioning resides in the availability of different forms of ROS with different molecular properties. The variation of chemical properties, mobility, half-life, and their capacity of interaction (Table 8.1) makes ROS the versatile molecule in signaling network (Suzuki et al. 2011; Bhattacharjee 2012b). Another key advantage of ROS functioning is tight link of cellular homeostasis with ROS metabolism. Any change in external environmental conditions that alter cellular homeostasis could lead to a change in the steady-state level of ROS which is subsequently being sensed by various redox-sensitive sensors to control metabolism. Therefore, the link between altered metabolism and ROS level under the exposure of unfavorable environmental cues would make ROS good signals to monitor changes in gene expression and metabolism that ultimately determine the ability of plants to survive under stress. In this chapter an effort has been made to provide an update of recent findings related to ROS-associated acclamatory mechanism and integration of ROS with other hormonal signaling, redox sensing, and gene expression.

8.2 ROS and Acclamatory Stress Tolerance in Plants

Plant adapt to environmental stresses through specific genetic responses. Molecular mechanisms associated with acclamatory stress tolerance, leading to the expression of genes as an early stress response, are largely unknown. However, it became gradually evident that the gene expression associated with acclamatory responses is highly sensitive to the redox state of the cell.

Plant cells have embraced the potential interactions with oxygen for metabolic regulations (Foyer et al. 1997; Suzuki et al. 2011). Surprisingly, ROS are important metabolites which participate in metabolism, growth, and morphogenesis of plant cells. The imposition of abiotic and biotic stresses can further increase the level of ROS (Arora et al. 2002; Bhattacharjee 2005, 2012b; Alscher and Hess 1993; Alscher et al. 1997; Suzuki et al. 2011). ROS are therefore implicated in most, if not all, stress responses. Being highly reactive, most of the ROS can cause membrane damage and inhibit enzyme activities, and therefore when accumulated, they are not compatible with cell function and considered to be deleterious and harmful. While O_2^-, $OH^•$, and $^1O_2$ have very few well-characterized role in plant cells, except perhaps in senescence, H_2O_2 may have important metabolic roles (Dougles 1993; Levine et al. 1994; Karpinski et al. 1997).

The steady-state level of ROS in a cell is largely determined by the efficiency of the antioxidative systems (Foyer et al. 1997; Foyer et al. 1994. When the production of ROS exceeds significantly than the capacity of the tissue to scavenge them, oxidative stress is favored. Much of the injury caused by the exposure of the abiotic and biotic stresses is associated with oxidative damage at cellular level. Augmentation of antioxidative defenses, therefore, plays a pivotal role in preventing stress-induced injuries and toxicities. Various efficient low-molecular-weight antioxidants and quenchers like glutathione, ascorbate, together with the activities of the antioxidant enzymes, are generally increased in plants under stressful conditions and correlate significantly with enhanced tolerance (Alscher and Hess 1993; Allen 1995). However, little evidences are available on the molecular mechanisms underlying the induction of defense genes.

There happens to be many putative bona fide signal transducing molecules under stress, for example, ethylene, ABA, and salicylic acid (Suzuki et al. 2011; Bowler et al. 1992; Kim et al. 1996; Mittler and Zilinskas 1992). Surprisingly, ROS like H_2O_2, $^1O_2$, and antioxidant molecule glutathione make important contributions to the redox state of the plant cell and are implicated in the activation of the genes that lead to the acclimation, stress tolerance, and other defense

responses (Alvarez et al. 1998; Foyer et al. 1997; Delgado-Lopez et al. 1998). It becomes gradually clear that gene expression associated with acclamatory stress responses is largely sensitive to the redox state of the cell. Of the many components that contribute to the redox balance of the cell, thiol/disulfide exchange reactions, particularly involving glutathione pool and the generation of ROS like H_2O_2, are the central components of the signal transduction in both environmental and biotic stresses.

Since H_2O_2 is an endogenous oxidant, with moderately higher half-life and diffusible, that accumulates in many stress situations (Alscher and Hess 1993; Foyer et al. 1997; Okuda et al. 1991), a central role for this metabolite as a diffusible signal for selective induction of defense genes has been envisaged (Alscher and Hess 1993; Chen et al. 1993a).

8.2.1 ROS in Systemic Acclimation to Photooxidative Stress

In higher plants, dissipation of excess photochemical energy (EPE) is an immediate and finely tuned response which occurs through heat irradiation, alternative sinks for photosynthetic electrons, and ultimately downregulation of Photosystem II (Foyer 1996; Huner et al. 1998; Foyer and Noctor 2009). The photoreduction of molecular O_2 is an alternative sink, especially when there is an acute dearth of $NADP^+$. But the spilling of electron to molecular O_2 is always associated with the formation of ROS, such as $O_2^{\bullet-}$, H_2O_2, $OH^\bullet$, and $^1O_2$. (Alscher and Hess 1993; Foyer 1996). If accumulation of ROS under conditions of EPE exceeds the capacity of enzymatic and nonenzymatic antioxidant systems to scavenge them, then photooxidative damage to photosynthetic apparatus ensues (Mehler reaction), which leads to cell death. This is manifested at whole plant level by the appearance of chlorotic lesions on damaged leaves. However, ROS may also play a positive role in response to EPE by initiating an increase in rate of degradation of DI protein, a key component of LHCII (Fig. 8.2). This causes photoinhibition of photosynthetic electron flow, which may be a protective mechanism in such conditions. The possibility of both positive and negative roles for ROS under EPE suggests that it is more appropriate to view equilibrium between the processes that produce ROS and antioxidative defense which destroy them rather than the levels of these antagonists per se (Foyer 1996). However, the immediate responses to EPE may lead to a whole plant acclimation which could include an alteration to the photosynthetic capacity of new leaves in which ROS also could play a role (Foyer et al. 1997; Huner et al. 1998). Therefore, in natural environment, how well plants tolerate different abiotic stresses may be determined by their ability to deal with EPE before ROS seriously poses problems to cellular structures especially chloroplasts (Krause 1994).

Several works highlights the impact of oxidative stress in response to fluctuating environmental conditions which elicit EPE on acclamatory responses of plants (Foyer et al. 1997; Krause 1998; Russell et al. 1995). When a leaf experiences a set of conditions that promote EPE, such as EL (Excess Light) conditions, the immediate response is an intracellular signaling of antioxidant defense genes elicited by redox changes in the proximity of PS II, which still followed by a subsequent increase in H_2O_2 level. Prolonged exposure under such condition leads to the death of such leaves. However, leaves suffering EPE also produce a systemic signal, a component of which is H_2O_2, which set up an acclamatory response in unstressed regions of the plant. The signaling, mediated by H_2O_2, leads to an increased capacity to tolerate further episodes of EPE-induced photooxidative stress by remote activation of antioxidant defenses, i.e., systemic acquired acclimation (Karpinski et al. 1999).

Experimentally, excess light (EL) applied to low light (LL) adopted *Arabidopsis* for up to 1 h causes rapid EPE and subsequently a burst in the titer of ROS, leading to the reversible photoinhibition (Santos et al. 1996). Surprisingly it was found that such a chloroplast-localized oxidative stress only induced genes encoding key components of cytosolic ROS-scavenging systems. One of these genes, APX 2, an ascorbate peroxidase isoform, is induced only under EL. The induction

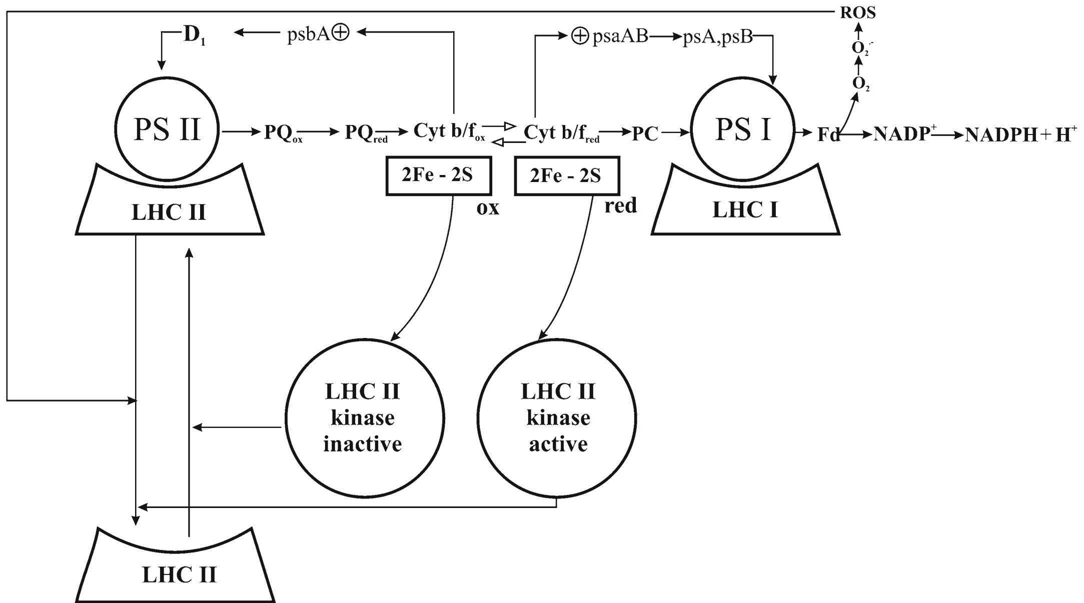


Fig. 8.2 Redox-sensing and transcriptional regulation of gene in chloroplast. In presence of excess photochemical energy (*EPE*), plastoquinone became predominantly reduced (PQ_{red}), resulting in the activation of LHCII kinase via structural changes around Rieske center (2Fe-2S) protein of cytochrome bf complex. The kinase in turn phosphorylates LHCII and PSII, resulting in migration of LHCII away from PSII and subsequently reducing light absorption by PSII. Oxidation of PQ_{red} reverses structural changes of cyt bf and leads to kinase inactivation. Phosphatase-mediated dephosphorylation of mobile LHCII leads to reassociation of PSII and further increase in light energy by PSII. Alternatively, redox state of PQ controls adjustment of stoichiometry of PSI and PSII by transcriptional regulation of chloroplastic genes that encode apoprotein PSI (psA and psB proteins) and PSII (D_1 proteins) reaction centers. ROS produced under EPE utilization may cause physical separation of PSII from LHCII by degrading D_1 proteins, thus reducing light energy absorption

of this nuclear gene is also found to be regulated by changes in the activity of PS II by rapid changes in the redox status of plastoquinone pool (PQ). Moreover, the major cellular antioxidant glutathione (Noctor et al. 1998) blocked the induction of this gene by EL, implying that redox change in the cellular redox pool may play a role in chloroplast to nuclear communication.

Treatment of leaves with H_2O_2 and then exposure to EL caused significantly greater induction of APX2 than control EL alone. This surprising observation was investigated in more detail in a series of time course experiments which revealed that detached leaves pretreated with H_2O_2 showed a slower decline in maximal PS II efficiency under EL conditions than control leaves, indicating that prooxidant status of leaf may be a crucial factor in adapting EPE. Protective effects of H_2O_2 have been described for maize seedlings, chilled in dark (Krause 1998), and have been explained by triggering various stress defense mechanisms (Foyer et al. 1997).

Though all those data do not indicate whether H_2O_2 has a direct or indirect effect, but they strongly emphasize a role of this ROS in the acclimation to conditions which invoke EPE. The opposite effects of exacerbated PS II inhibition upon treatment with antioxidant GSH (Foyer et al. 1997), which reduces H_2O_2 by enzymatic and nonenzymatic reactions, are consistent with such a role of H_2O_2.

8.2.2 Organeller Redox Signaling for Stress Acclimation

The redox state of organelles like chloroplast, mitochondria, and peroxisome, manifested through reductant/oxidant pool of metabolites and carrier proteins, is largely involved in the

regulation of several biological processes including hormone signaling, nuclear and chloroplastic gene expression, and ultimately the stress acclamatory performances (Suzuki et al. 2011; Foyer and Noctor 2009; Pfannschmidt et al. 1999). The expression of nuclear gene-encoded chloroplastic proteins has been found to correlate with the redox status of plastoquinone, glutathione, and ascorbate pool in coordination with environmental stress and chloroplast development (Foyer and Noctor 2009). The changes in expression of nuclear antioxidant and defense genes are found to be correlated with excess photochemical energy (EPE)-induced changes in redox state of plastoquinone pool in chloroplast (Karpinski et al. 1997; Li et al. 2009). In this regard chloroplastic kinases and phosphatases play vital role in the regulation of redox state of regulatory proteins under environmental stress (Pesaresi et al. 2010). The transitional balance that regulates Z-scheme photosynthetic energy distribution and fluxes is found to be largely mediated by plastoquinone (PQ) pool (Dietzel and Pfannschmidt 2008). Any imbalance in energy fluxes of Z-scheme of photosynthesis between PSII and PSI is also balanced and maintained by adjusting photosystem stoichiometry through changing abundance of LHCPs and reaction centers (Pesaresi et al. 2010) in both short- and long-term responses. Protein kinases STN7 and STN8 found to play crucial role in redox regulation of PQ and quantitative phosphorylation of PSII (Pesaresi et al. 2010; Bonardi et al. 2005).

Recent proteomic studies also revealed the role of phosphorylation of mitochondrial redox proteins in mitochondrial redox regulation for stress acclimation mechanism involving mtETC complexes, ATP synthesis, and TCA activity (Ito et al. 2009). More identification of mitochondrial protein kinases/phosphatases as well as their target proteins is required for complete unfolding of the process of redox regulation and signaling in mitochondria.

8.2.2.1 Redox Retrograde Signaling and Stress Acclimation

The mechanism of retrograde signaling (Organelle to nucleus) has evolved to communicate and intricately coordinate metabolism and gene expression between organelle and nucleus (Fig. 8.3) that subsequently control the reverse process (nucleus to organelle) signaling (Woodson and Chory 2008; Suzuki et al. 2011). Both the developmental control of organelle biosynthesis and operational control of stress acclimation are regulated by retrograde signaling in plants. The redox state of chloroplast, mitochondria, and peroxisome (which depend primarily on the titer of ROS and antioxidants) are recognized as primary retrograde signals that play pivotal role in stress acclimation mechanism (Pogson et al. 2008; Suzuki et al. 2011). Any changes in redox status of these organelles, particularly under unfavorable environmental cues, can be sensed and transmitted to nucleus by retrograde signaling cascades involving primary redox-sensitive proteins and/or by metabolic coupling (Galvez-Valdivieso and Mullineaux 2010).

Chloroplast is extremely prone to oxidative damage, primarily due to availability and chemistry of oxygen. This is particularly the scenario when the rate of Z-scheme exceeds the capacity of regeneration of $NADP^+$ by photosynthetic carbon reduction cycle (PCRC) under EPE. The dearth of $NADP^+$ under this situation compels the movement of electrons of Z-scheme of photosynthesis to reduce O_2 partially, leading to the generation of superoxide and other toxic ROS, which subsequently causes photooxidative damages. Retrograde signaling has been studied in seedling treated with norflurazon (inhibitor of carotenoid biosynthesis that causes photooxidative damages) or mutants with underdeveloped chloroplasts (Nott et al. 2006; Koussevitzky et al. 2007). Three different conditions were shown to induce the production of chloroplast derived signals in *Arabidopsis* and their dispatch to nucleus, altering the expression of nuclear genes, depending on the presence of GUN1 in chloroplast and AB14 in nucleus:

(a) Accumulation of Mg-protoporphyrin IX (chlorophyll biosynthesis intermediate) and its methyl ester, which alters gene expression in *Arabidopsis*
(b) Inhibition of plastid gene expression at translational stage of protein synthesis
(c) Changes in the redox state of photosynthetic electron carrier

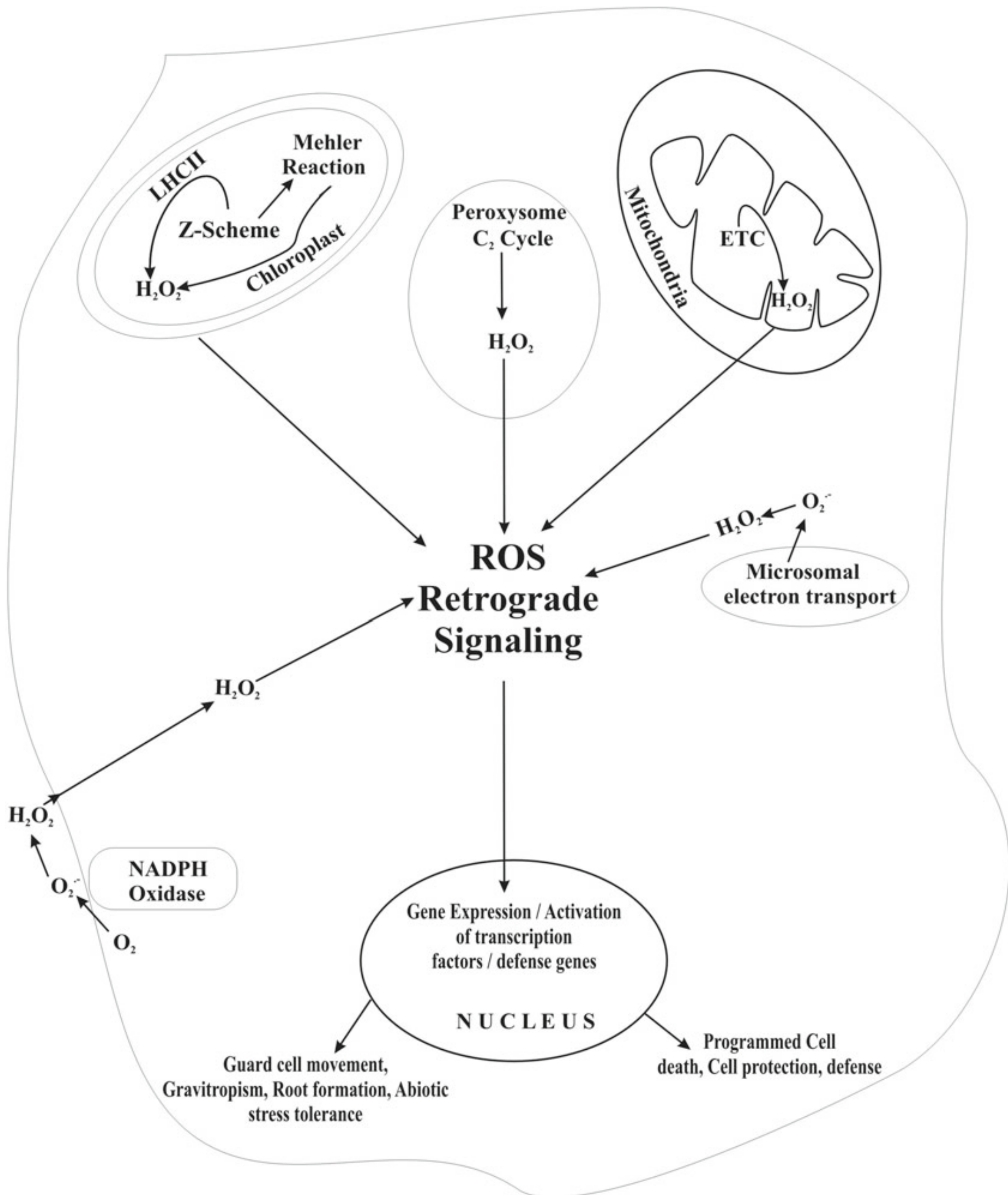


Fig. 8.3 ROS generation in different locale and subcellular organelle of photosynthetic green cell. ROS produced by Mehler reaction in chloroplast, ETC in mitochondria, C_2 cycle in peroxisome, microsomal electron flow, and specific enzyme-mediated reactions (NADPH oxidase) acts in redox retrograde signaling where the oxidant signal is transduced from organelle to the nucleus for triggering gene expression and downstream physiological events like stress tolerance, guard cell movement, programmed cell death, pathogen and defense

ROS-dependent retrograde signaling is assumed to be primarily used for stress signaling as integrated acclamatory responses of plants (Woodson and Chory 2008). In fact, GUN1 and AB14 retrograde signaling pathways found to have great influence on plant performances under abiotic stress and their acclimation.

The most important ROS that initiate retrograde signaling pathway in plant is $^1O_2$, which is even found to evoke their activity irrespective of Mg-protoporphyrin IX and GUN1-mediated signaling. $^1O_2$ signaling pathway has been extensively studied in *Arabidopsis* using flu mutants that accumulate protochlorophyllide (potent photosensitizer) during dark adaptation and which also generate $^1O_2$ upon reexposure to light (Lee et al. 2007). Using this mutant of *Arabidopsis* and employing transcriptome analysis and DNA microarray, several workers have identified distinct set of genes specifically activated by $^1O_2$ (Gadjev et al. 2006).

There exist also the interaction between $^1O_2$ and other ROS like H_2O_2 as a variation in retrograde signaling. H_2O_2 has a positive role in reducing the probability of formation of $^1O_2$. Application of H_2O_2 has shown to promote the oxidation of quinone A, thereby increasing the photosynthetic electron transport and reducing the generation of $^1O_2$ under stress (Asada 2006). So, *Arabidopsis* flu mutants, overexpressing tAPX (thylakoid ascorbate peroxidase), strongly reduce the activation of nuclear gene expression through $^1O_2$ retrograde signaling pathway.

8.3 Evidences of Role of ROS and Oxidative Burst in Stress Acclimation

The oxidative burst, during which large amount of ROS are generated, is now well established as one of the earliest responses of plant cells under abiotic and biotic stresses (Arora et al. 2002; Bhattacharjee 2005; Foyer et al. 1997; Miller et al. 2008). H_2O_2 generated during this oxidative burst has several effects: it can mediate oxidative cross-linking of cell wall polymers, it may be directly microbiocidal, it can induce the expression of inducible genes encoding proteins involved in defensive and antioxidative responses, and even it can induce programmed cell death characteristic of hypersensitive responses (Alveraz and Lamb 1997; Neill et al. 1999; Sakamoto 2008). The role of H_2O_2 as a signaling molecule mediating acquisition of tolerance to both biotic and abiotic stresses has became clear from several works (Foyer et al. 1997; Smirnoff 1998; Bhattacharjee 2012a, 2013). There are several reports of cross-tolerance and some cellular responses have been found to be common to a number of different stresses (Foyer et al. 1997). An inductive pulse of H_2O_2 is required to switch on some stress acclamatory metabolism (by upregulating antioxidative defense and restoring redox homeostasis), through which plant prevents or repairs oxidative damages to newly assembled membrane system caused by unfavorable environmental cues during early germination in two rice cultivars (Bhattacharjee 2012a, 2013).

It is possible that different stresses induce both common and distinct or unique responses. However, the particular spectrum of responses determines the output. For example, H_2O_2 may be generated during several stresses but may only reach threshold concentration in certain situations or may synergize with other molecules such as salicylic acid and nitric oxide to exert its effect (Dangl 1998). Therefore the key requirement is to identify the signaling pathways induced by ROS and to determine how they mediate acclimation to various environmental stresses.

Application of reactive oxygen species (ROS), specifically H_2O_2, can induce stress tolerance in plants. Treatment of winter wheat with low concentrations of H_2O_2 and inhibitor of catalase induced the synthesis of polypeptides similar to those synthesized under chilling stress (Matsuda et al. 1994). Prasad et al. (1994) also reported that *maize* seedlings became more chilling tolerant following pretreatment with H_2O_2. A transient increase in H_2O_2 was suggested to activate signal activation of protective mechanisms for acclimation to chilling (Prasad et al. 1994, 1995; Neuenschwander et al. 1995). Doke et al. (1994) proposed that H_2O_2 generation should be

considered as a central trigger for defense metabolism following exposure to abiotic and biotic stresses. H_2O_2 also found to be responsible for the expression for chilling responsive genes (Neuenschwander et al. 1995). In *Arabidopsis*, treatment with H_2O_2 altered cytosolic calcium concentrations similar to those observed during cold acclimation (Knight et al. 1996).

In numerous studies, suspension cultures of *Arabidopsis thaliana* is used as model system to explain the signaling processes required for both the generation of H_2O_2 and subsequent cellular responses that it induces. Treatment of such cultures with harpin, a proteinaceous bacterial elicitor, induces rapid oxidative burst that requires both protein phosphorylation and calcium influx (Desikan et al. 1997; 1998b). In this case, as suggested by Doke et al. (1994), H_2O_2 arises primarily from dismutation of O_2^-, which is formed via single electron reduction of molecular O_2 catalyzed by a plasma membrane – located enzyme similar to NADPH oxidase. In *Arabidopsis* suspension cultures both biochemical and pharmacological evidences are consistent with the activity of NADPH oxidase (Desikan et al. 1996) and homologues of gp91, the key redox component of the enzyme complex (Desikan et al. 1998a).

Various lines of evidence support the existence of cross-tolerance, i.e., induction of tolerance to a particular kind of environmental stress involving oxidative stress that also increases the tolerance to one or more other kinds of stresses including biotic stresses. For example, O_3 exposure to *Arabidopsis thaliana* induces resistance to virulent *Pseudomonas* strains (Sharma et al. 1996). In this case O_3 exposure resulted in the expression of a number of pathogenesis-related (PR) proteins and genes. Similarly cotton plants exposed to water deficit were found to be more resistant to paraquat than water deplete plants (Burke et al. 1985). All these results provide evidence supporting the hypothesis that common redox signals are involved in the induction of acclamatory responses to both abiotic and biotic stresses. Evidences for the involvement of H_2O_2 and GSH in the signal transduction and regulation of gene expression is provided by reports of acquisition of stress tolerance by exposure to abiotic stresses with accompanying changes in gene expression (Foyer et al. 1997, Bowler et al. 1992).

8.4 Redox-Sensing, ROS-Mediated Signal Transduction, Their Targets and Stress Acclimation

The molecular mechanisms associated with signal transduction, leading to changes in gene expression, are one of the early stress responses and are extremely complicated. It is clear, however, that gene expression associated with acclamatory responses is sensitive to redox state of the cell. Of the many components which contribute to the redox balance of the cell, two factors have been shown to be crucial in mediating stress responses. Thiol/disulfide exchange reactions, involving glutathione pool and the generation of ROS, particularly H_2O_2, are the central components of signal transduction in both abiotic and biotic stresses.

Redox signals are the most fundamental forms of information monitored by the plants (Mahalingam and Fedoroff 2003; Suzuki et al. 2011). More complex aspects of redox control of physiology of plants ultimately through the regulation of gene impression developed with the evolution of higher plants. It is now widely accepted that redox signals are the key regulators of plant metabolism, growth, and development and may even have plenty of cross talking with other system of signal transduction of parts. In fact controlled generation of ROS acts as “second messenger” along with other mediators like Ca^{2+}, not only in plant responses to environmental stress but also in hormone signaling (Shapiguzov et al. 2012; Suzuki et al. 2011; Varnová et al. 2002).

To have a comprehensive idea regarding the ROS-mediated signal transduction, one has to develop the primary understanding of the fact on how the increased titer of ROS is sensed. One simple possibility is the direct modification of transcription factor with redox-sensitive groups (Allen 1993). Chemistry of ROS sensing dictates

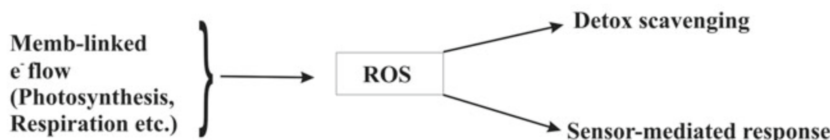
that the redox-sensing proteins have a commonality, with active thiol groups as potential ROS target. But, in reality there seems to be much more complex signal transduction routes. In fact, the ROS-associated redox changes in the different organelles (mainly chloroplast and mitochondria) are signaled to the nucleus. Surprisingly the "sensing" function is generally performed by the antioxidative system itself. This system acts as a strong buffer against ROS, maintaining relatively low concentrations of oxidants under normal optimal conditions. The redox balances of the cell are generally perturbed without large changes in the concentration of ROS. This is feasible because of the presence of plethora of components capable of scavenging ROS. In case of catalase mutants placed under photooxidative stress or excess photo chemical energy, H_2O_2 concentration is not greatly increased relative to the wild type, but the glutathione pool is found to be massively perturbed (Allen 1993). The enhanced availability of ROS may therefore be sensed by the cell as increased oxidative flux through key components, rather than marked increase ROS titer. This view strongly supports the existence of a dynamic system for allowing acclamatory changes through the components of antioxidative system that are integrated into the signal transduction network. It would allow appropriate response to occur as a result of increased flux of ROS, even in absence of mark changes in ROS titer. Apart from that, changes in ROS trigger marked modulation in the expression of gene far beyond their perception by antioxidative systems (Mahalingam, and Fedoroff 2003). This ultimately confers upregulation of defense system against environmental stresses. Many studies strongly support the view that some antioxidative compounds have dual role of scavenging and signaling (through sensor scavenging). In these cases the antioxidants probably exhibit low capacity of antioxidative role compared to classical detoxification scavenging (Fig. 8.4).

Studies related to the nature of "sensor-scavenging component" revealed that heme-based enzymatic antioxidants such as catalase and ascorbate peroxidases and thiol-containing antioxidant molecules such as GSH are basically the candidates for such responses. Plants also contain numerous proteins with redox-active thiol groups, some of which have been shown to have activity against peroxides. These include chloroplastic and cytosolic glutathione peroxidases, chloroplastic and cytosolic peroxiredoxins, and glutaredoxins and chloroplastic thioredoxins (Rouhier et al. 2009; Nishiyama et al. 2001; Kunert and Foyer 1993). Although the exact roles of these thiol-containing sensor scavengers remain to be elucidated, it is clear that there may be considerable divergence of function within each class of these molecules.

YAP-1 is a basic zipper type transcription factor which induces several genes in response to H_2O_2. Peroxides enhance the accumulation of nuclear YAP-1 by oxidizing cysteine residue from intramolecular disulfide bond that appears to trap YAP-1 in the nucleus, thereby enhancing their reactivity (Kuge et al. 1997). *Arabidopsis* genome does not appear to contain sequence similar to YAP-1, but there may well be functionally similar elements in the initial perception of changes in redox state of plant.

Peroxiredoxins (PRX), otherwise known as thioredoxin (THX) peroxides, have been shown to reduce peroxides using reductant from thioredoxins (Trx). The enzyme system is closely linked to Z-scheme of photosynthesis via a specific chloroplastic Trx isoform (Rouhier et al. 2009). In fact the primary role of these thiol-containing redox-sensitive proteins is not to detoxify peroxides but to sense enhanced production or in other words to initiate a signaling process by acting as a "signal-scavenging" component (Fig. 8.4).

Another mechanism of redox-signal perception involves oxidation of glutathione pool accompanied by increase in total glutathione under environmental stresses (Rouhier et al. 2009). The method of sensing this redox perturbation involves protein glutathionylation, in which GSH forms a mixed disulfide with target protein, thereby modifying the activities of enzymes and transcription factors. This process is considered to play an important role in redox signaling and protection of protein structure and function.



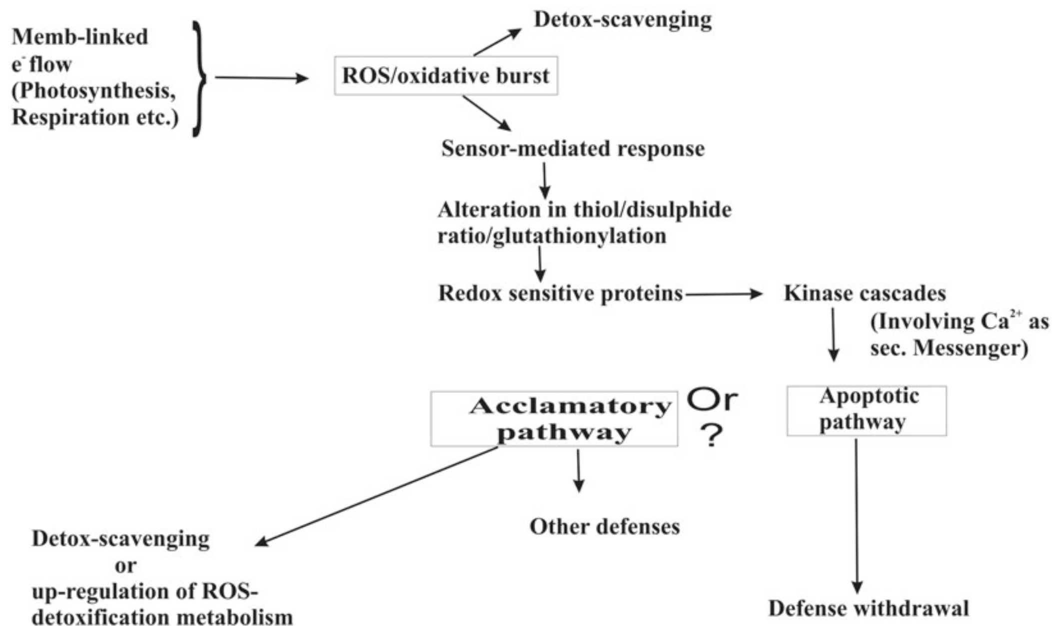


Fig. 8.4 ROS, antioxidant, and redox signaling network in plants under normal and unfavorable environmental conditions. Model explaining the perception of enhanced ROS involving redox components or antioxidant system. Under normal growing condition (I), ROS are produced as a consequence of many metabolic events and are efficiently removed by detox scavenging of antioxidants. However, under stress (II) increased production of ROS or oxidative burst causes increased oxidation of "sensor-scavenging" redox components locked into signal transduction pathways. Signaling pathway operates via kinase cascades and other secondary messengers that ultimately lead to upregulation of ROS detoxification capacity (acclamatory pathway) or cell death pathway involving defense withdrawal. The decisive factors that determine the feasibility of acclamatory pathway or cell death pathway could be involved to determine the intensity of oxidative stress or ROS signal and their location

Another significant class of thiol-containing protein is certain subclasses of GST superfamily which is active in reducing peroxides or dehydro-ascorbates (DHA) or in catalyzing thiol transferase reaction (Daniel 1993). Some of these GSTs are strongly induced by oxidative stress especially under biotic stresses. So, it seems that the power of ROS can be effectively harnessed (as most of these are short lived) to convey redox information. These signals in most of the cases are incorporated into complex redox network that involves electron carriers (plastoquinone, ubiquinone) and electron acceptors (like TRX, Fd).

8.4.1 Redox-Sensitive Proteins and Redox Signaling

Plant cell can sense, transduce, and translate the ROS signals into appropriate cellular responses

through the involvement of redox-sensitive proteins. Redox-sensitive proteins mainly operate through reversible oxidation/reduction thereby switching "on" and "off" depending on the cellular redox state. ROS can oxidize the redox-sensitive proteins directly (Nishiyama et al. 2001) or indirectly via some other ubiquitous redox-sensitive molecules like glutathione or thioredoxins which control the cellular redox state (Rouhier et al. 2009). Therefore, redox-sensitive proteins are susceptible to oxidation and reduction that depends on the titer of ROS and or redox state of the cell. Redox-sensitive proteins further execute their function via downstream signaling components like kinases, phosphatases, and transcription factors. In some cases ROS directly oxidize the target proteins, particularly peroxiredoxins and thioredoxins, and subsequently the transcription factors (Rouhier et al. 2009; Nishiyama et al. 2001). In fact, most of the redox regulation of gene expression is mediated by a family of protein disulfide oxidoreductases, namely, thioredoxins, peroxiredoxins, glutaredoxins, and protein disulfide isomerases (Rouhier et al. 2009; Imsande 1999). Thioredoxins are small (approximately 12 kDa) protein with S = S reducing activity. They oxidized directly by ROS or indirectly by peroxiredoxins (Thioredoxin peroxidase). Thioredoxins may be reduced by thioredoxin reductase and by NADPH-dependent enzymes. There are ample of evidences to suggest that thioredoxins and other similar proteins are enzymatic mediators of the regulatory effects of ROS at transcriptional levels (Mahalingam and Fedoroff 2003). It is found that UV irradiation promotes translocation of thioredoxin to the mammalian nucleus where it activates stress-related transcription factors like NF-kB and AP-1 by enhancing DNA binding. Thioredoxin can bind directly with NF-kB p^{50} and interacts indirectly with AP-1 through redox factor 1 (Mylona and Polidoros 2010). On the other hand, plants possess chloroplastic, mitochondria, and cytosol redox-regulating system for controlling expression of genes (Mahalingam, and Fedoroff 2003; Rouhier et al. 2009). Ferredoxin, being the component of PS I of Z-scheme of electron flow, gets photo-reduced during photosynthesis. The reducing power of Fd is then subsequently transformed to Thioredoxin by Fd-Trx reductase, which then interacts with target enzymes. There are distinct classes of "thioredoxin target" including the "transcription factors." One of the best studied redox-regulatory plant proteins is a class of RNA-binding proteins that control translation or stability of chloroplastic mRNA under the exposure of photochemical energy by way of ferredoxin-thioredoxin system (Rouhier et al. 2009).

8.4.2 ROS and Redox-Regulated Gene Expression

Identification of changes in gene expression regulated by oxidative stress is of considerable importance for developing stress tolerant plants. However, till date, a global analysis of the effect of ROS on the transcriptome of any one plant species has not yet been completely described. The information regarding regulatory role of ROS on gene expression is primarily the outcome of experiments using mutant and transgenic plants with direct application of ROS or ROS-generating chemicals. Results of these experiments contributed information on ROS-responsive inducible genes. In recent times, a number of genes involved in signal transduction, stress acclimation and defense, metabolism, and cell structure have been identified, ultimately revealing a highly dynamic and redundant network of ROS-sensitive genes (Allen 1995; Mylona and Polidoros 2010).

In bacteria ROS induces expression of at least 80 genes (Demple 1991). In yeast approximately 300 genes (Godon et al. 1998) and in plants more than 100 genes are found to be induced by ROS (Stephen et al. 1995), and the numbers are growing with the application of cDNA microarray technique to carry out a transcriptomic analysis of oxidative stress-regulated gene expression (Varnová et al. 2002). Bacterial genes are organized in regulons that are controlled by specific transcription factors. O_2^- -induced genes are controlled by the Sox R protein that has Fe–S cluster and upon oxidation induces the expression of a

downstream transcription factor called Sox S. H_2O_2-induced genes are controlled by the oxidation of thiol groups present in the transcription factor Oxy R (Storz and Imlay 1999). Alternatively, two-component systems may activate the expression of bacterial genes upon changes in the redox status of the cell. A redox sensor, a membrane-associated phosphoprotein, becomes phosphorylated on histidine when it is either oxidized or reduced by components of electron transport chain. Its substrate, the redox response regulator, is a sequence-specific DNA-binding protein that is phosphorylated at an aspartate residue that regulates transcription (Allen 1993). In yeast, genes induced by redox signals consist of a complex network of different regulons, so-called stimulons (Jamieson 1998). Activities of one of the best studied redox-sensitive transcription factors, yAPI, are found to be controlled by redox signals at the level of nuclear localization and DNA binding (Kuge et al. 1997). Induction of oxidative stress causes relocalization of yAPI from cytoplasm to nucleus, and its DNA-binding capacity increases many-fold. In mammalian systems, many studies point to the significance of two classes of transcription factors that are sensitive to redox signals: the nuclear factor κB (NF-κB) and the activator protein-1 (AP-1). The prooxidant state in cytoplasm (largely determined by the ratio of GSSG and GSH) or ROS activates these transcription factors and induces their mobilization to nucleus, where a reducing environment is required for proper DNA binding. Thioredoxins and redox factor Ref-1 provide the reducing power for DNA binding (Arrigo 1999). Therefore, two major steps in transcriptional activation of eukaryotic transcription factors seem to be influenced by redox balances: nuclear relocalization and DNA binding.

In plants, generation of ROS occurs under diverse range of conditions, and it appears that ROS accumulation in specific tissues and appropriate quantities is of benefit to plants and can mediate cross-tolerance towards other stresses. ROS, specifically H_2O_2, is found to be involved in plant defense response, affecting both gene expression and activities of proteins such as MAP kinase, which in turn functions as regulators of transcription (Desikan et al. 1999, 2000). In spite of the fact that ROS and cellular redox state are known to control expression of plant genes, the signaling pathway(s) involving transcription factors or promoter elements specific for the redox regulation are still to be identified. There are, however, several candidates for promoter elements as well as DNA-binding factors that act as redox response elements (Fig. 8.5).

One of the examples of induction of defense genes, controlled by redox balance of the cell is Glutathione S-transferase (GSTs), which catalyzes the conjugation of GSH to a variety of hydrophobic electrophilic compounds that otherwise attack important cellular macromolecules (Marrs 1996). Compounds with bound GSH undergo cellular detoxification pathway (Daniel 1993). The signal by which the expression of GST gene is regulated is believed to be a prooxidant state of the cells, probably resulting from a reduced GSH content (Daniel 1993). The promoter element responsible for the induction of the Ya subunit in mouse GST by electrophilic compounds consists of two adjacent AP-1-like sites (Friling et al. 1992). The consensus sequence of this site is ΔGACA (A/T) (A/T) GC and is called an antioxidant-responsive element or electrophile-responsive element. Two adjacent AP-1-like sites are also present in the *Arabidopsis* GST 6 gene and constitute promoter element (Chen et al. 1996). This promoter element is at least, in part, required for GST 6 inductions by H_2O_2, SA, and auxins (Chen and Singh 1999). A single antioxidant-responsive element has recently been identified in the promoter of a maize catalase gene (Cat 1) and was found to bind molecular factors from senescing scutella that accumulate Cat 1 transcripts, probably as a result of oxidative stress (Polidoros and Scandalios 1999; Dat et al. 2000).

The G box (CACGTG) is a ubiquitous cis-element present in many plant genes and is thought to mediate response to diverse environmental stimuli, including primarily redox changes (Dröge-Laser et al. 1997). Together with the H box (CCTACC), the G box functions in the activation of phenyl propanoid biosynthetic genes. Transcription of at least two of these genes that encode phenylalanine ammonia-lyase

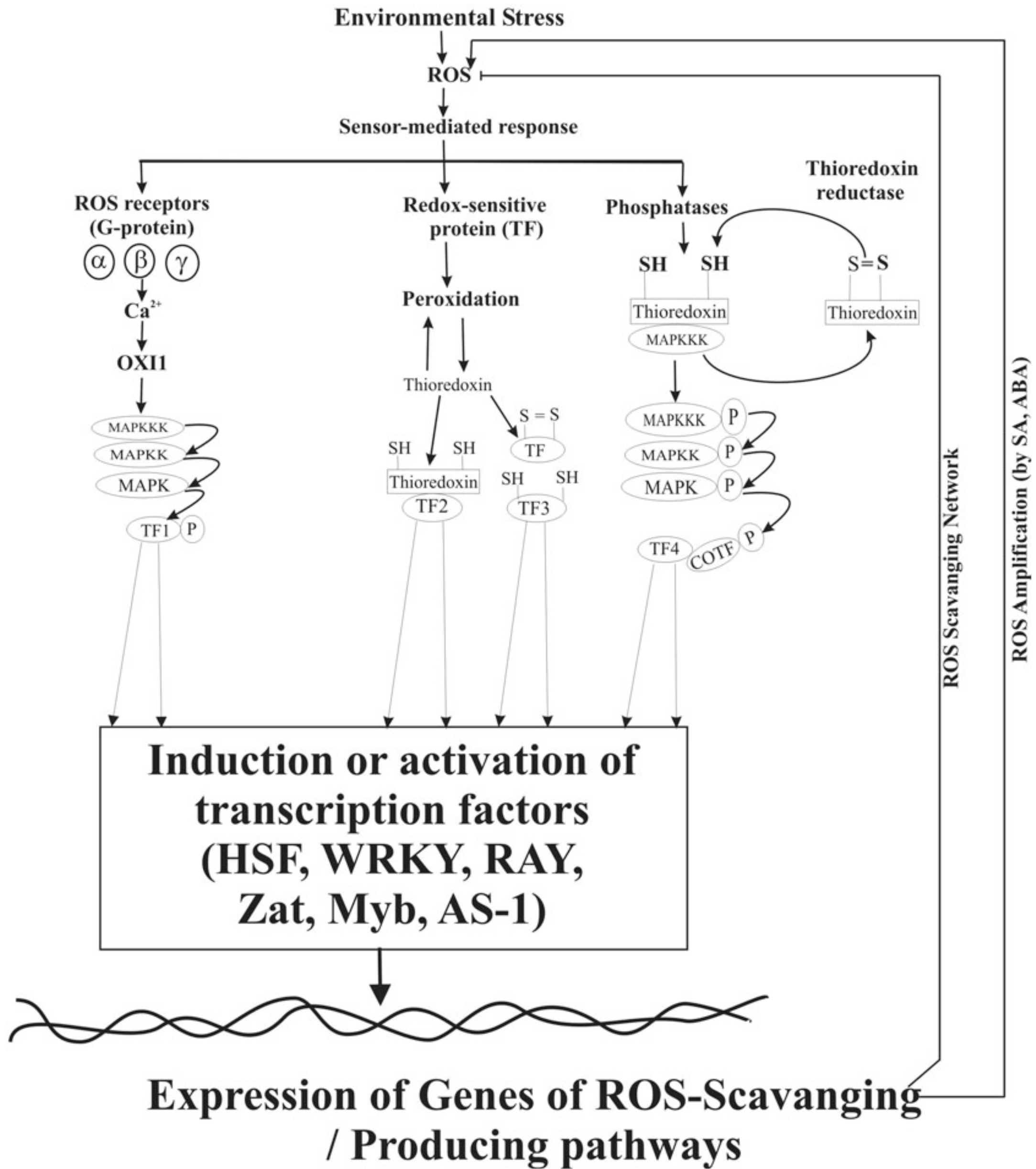


Fig. 8.5 Schematic pathways showing the role of reactive oxygen species (*ROS*) in gene expression (*ROS* reactive oxygen species, *MAPK* mitogen-activated protein kinase, *MAPKK* MAPK kinase, *MAPKKK* MAPKK kinase, *TF* transcription factor, *COTF* transcription cofactor)

(PAL) and chalone synthase (CHS) is under redox control (mainly controlled by GSH) (Wingate et al. 1988).

Heat shock elements and heat shock factors also participate in redox-regulated gene expression. Activation of heat shock factor is characterized by the conversion of this factor from monomer to trimeric state, a process induced by heat shock and large variety of conditions that generate abnormally folded proteins. Disulfide-linked aggregates of cellular proteins are formed as a consequence of disturbed

intracellular redox homeostasis and are one of the signals required for HSF trimerization (Arrigo 1999).

A novel homeodomain protein of HD – zip class isolated from tomato and zinc – finger protein LSD1 from *Arabidopsis* is negative regulators of oxidative cell death and has been proposed to act as transcriptional regulators downstream of ROS signal (Mayda et al. 1999).

8.4.3 *Arabidopsis* Transcriptome Analysis Under Oxidative Stress

Recently, it has been postulated that ROS levels are probably sensed by ROS-sensitive transcription factors in *Arabidopsis* (Miller et al. 2008; Miller and Mittler 2006). These so-called transcription factors or sensors are proposed to act upstream in a cascade regulating other transcription factors (like Zat family, Myb, HSF, WRKY transcription factor (Fig. 8.5)) and some stress-responsive protein as well (Miller et al. 2008; Mittal et al. 2009). Based on *Arabidopsis* model, it is now suggested that the generation of ROS signaling component leads to activation of transcription factors (Zat family, Myb, HSF, WRKY) and subsequently the expression of ROS-regulated genes. Such events ultimately establish molecular link through which cellular responses to different forms of abiotic stresses are related.

To analyze cellular response to ROS, several workers across the globe undertaken large scale analysis of *Arabidopsis* transcriptome during oxidative stress. Using cDNA microarray technology, Desikan et al. (2001) have identified 175 nonredundant expressed sequence tags that are regulated by H_2O_2. Treatment of *Arabidopsis* culture with 20 mM H_2O_2 for 1.5 and 3 h and subsequently isolation of mRNA from control (untreated) and H_2O_2-treated cells serve as probes for the *Arabidopsis* Functional Genomics Consortium Cycle (AFGCC) 1 microarray analysis. Ultimately a global representation of the changes in expression of all the expressed sequence tags (ESTs) on microarray became evident. For the vast majority of transcripts, expression appeared unchanged with H_2O_2 treatment. However, there were clear indications of 175 nonredundant ESTs with a change in expression greater than 1.5-fold in response to ROS (H_2O_2) treatment. Of these, expression of 113 was upregulated and 62 downregulated.

One of the genes identified via microarray analysis as being expressed at low levels but H_2O_2 responsive was that encoding a protein Tyr phosphatase. Protein Tyr phosphatases are important signaling enzymes that regulate protein phosphorylation in all eukaryotes, particularly the interaction of MAPK cascades (Luan 1998). Oxidative stress activates MAPK cascade not only in plants (Kovtun et al. 2000) but also in animals, where Tyr phosphatase have been identified as H_2O_2 inducible. An *Arabidopsis* protein Tyr phosphatase has previously been identified that is transcriptionally regulated by abiotic stress factors like cold and salt (Xu et al. 1998).

Calcium signaling involving Calmodulin has been implicated in sensing environmental cues and imparting stress tolerance. A Calmodulin gene was found to be strongly induced by H_2O_2 (Desikan et al. 2001). In *Arabidopsis* suspension culture, application of harpin (a proteinaceous elicitor), which induces oxidative burst, causes not only the rise in the level of intracellular Ca^{2+} and protein phosphorylation but also the expression of defense genes (Desikan et al. 2001). Furthermore, NADPH oxidase, a potential ROS-generating enzyme, contains EF had calcium-binding motifs (Keller et al. 1998) and at least one of the NADPH oxidase genes is induced by H_2O_2 (Himelbau and Amasino 2000). These observations suggest that H_2O_2 induction of a Calmodulin gene might at least, in part, be regulating the activity of this enzyme. Moreover, a Calmodulin has been shown to mediate between calcium and ROS generation undergoing hypersensitive response. Dehydration or desiccation stress-induced stomatal closure in plants also involves oxidative stress-induced Ca^{2+} signaling. It was demonstrated that ABA induces generation of H_2O_2 in stomatal guard cells and subsequently H_2O_2-activated Ca^{2+} influx and stomatal closure (Desikan et al. 1998a). Thus, a significant extent of cross-tolerance occurs between ROS and Ca^{2+},

where both these signaling intermediates mediate cross-tolerance to a variety of stresses (Bowler and Fluhr 2000).

Among the genes induced by H_2O_2 was one encoding a blue copper-binding protein, which might function to sequester Cu, a toxic heavy metal (Himelbau and Amasino 2000). This gene was also induced by harpin and UV and its expression was up- regulated in senescing leaves. Thus, it is not surprising that there are genes that are induced by both oxidative stress and senescence.

The expression of genes encoding a mitochondrial uncoupling protein, pyruvate decarboxylase, and a myb-related transcription factor were induced by H_2O_2 (Finkel and Holbrook 2000). Mitochondrial uncoupling proteins are key factors regulating ATP synthesis and generation of ROS in mitochondria, which in turn maintains the redox balance of the cell.

Some of the H_2O_2-sensitive genes could also be involved in plant signaling. For example, a gene encoding a syntaxin was identified as H_2O_2 responsive by both microarray and RNA blot analysis. Syntaxins are docking proteins involved in vesicle trafficking, and a role in the hormonal control of guard cell ion channels has been demonstrated for an ABA-inducible syntaxin in tobacco (Leyman et al. 1999). As both the elicitors and ABA induce H_2O_2 production in guard cells, it could be that induction of a syntaxin by H_2O_2 is involved in regulating guard cell functioning (Pei et al. 2000). Similarly, genes encoding myrosinase-binding proteins and JA-inducible proteins were shown to be H_2O_2 responsive on the microarray analysis (Reymond et al. 2000).

Transcription factors have been reported to be induced rapidly during defense responses (Durrant et al. 2000). Various genes encoding transcription factors under adverse environmental stresses have been found to be induced by ROS, particularly by H_2O_2, suggesting that these transcription factors mediate further downstream H_2O_2 responses. Among the transcription factors induced by H_2O_2, EREBP, and DREB2A are important ones that regulate gene expression during various environmental stresses (Riechmann and Meyerowitz 1998). The involvement of zinc finger transcription factors in stress responses has been reported. During barley-powdery mildew interactions, a zinc finger protein was identified as a key mediator of R gene-induced resistance responses such as H_2O_2 generation (Shirasu et al. 1999). A host of other kinds of stresses such as salinity, ozone, UV, and wounding also induce this class of genes (Takatsuji 1999).

H_2O_2 not only activates the expression of genes but also repressed the expression of some genes. Desikan et al. (2001) reported downregulation of expression of 62 genes in *Arabidopsis*. Many of these encode proteins of unknown functions. It is interesting to note that genes encoding a receptor protein kinase and cys-proteases were repressed by H_2O_2.

The microarray analysis has enabled us to identify a number of expressed sequences tags (ESTs) regulated by oxidative stress, which are of potential importance to diverse stress responses. Co-regulation of those genes by various stresses supports the hypothesis that H_2O_2 mediates cross-tolerance (Desikan et al. 2001; Bowler and Fluhr 2000). Coordinated expression of several genes in response to specific stimulus can be achieved via the interaction of transcription factors with cis-elements common to the promoter regions of those genes. For example, WRKY binding site was identified in the promoter region of 26 genes, making up the "pathogen regulon" in *Arabidopsis* (Maleck et al. 2000). Analysis of the 1.1-kb 5′-upstream region of all the oxidative stress-induced genes did not reveal the presence of unknown binding site common to them. However, Desikan et al. (2001) identified 5′-upstream regions in the H_2O_2-induced genes that are potential binding sites for redox-sensitive transcription factors. These included binding sites for myb (Myrset et al. 1993), Osc/As-1-like proteins (associated with auxin and SA-induced genes), and AP-19 (Qin et al. 2000).

The work done so far on *Arabidopsis* demonstrates that H_2O_2 can modulate the expression of a subset of genes within *Arabidopsis* genome and also potentially alter the activity of cellular proteins. The mechanistic roles of H_2O_2 by dint of which these changes are induced still remain to be elucidated. It may be possible that H_2O_2

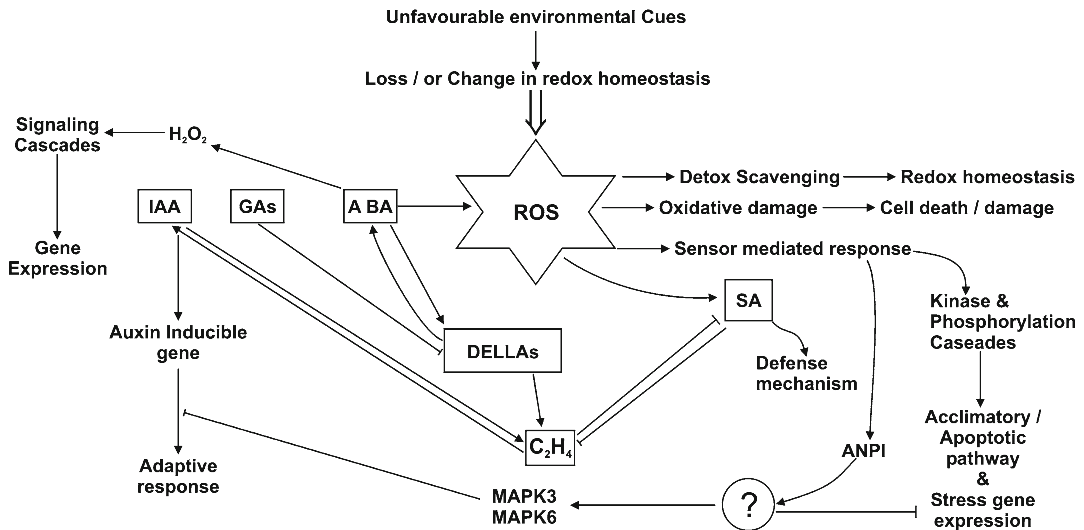


Fig. 8.6 A schematic model showing interactions (feed forward, backward, and inhibitions) between ROS and different signaling pathways, ultimately controlling stress acclamatory and adaptive responses under unfavorable environmental cues

can interact directly with the target proteins thereby altering the conformation of the proteins (Abate et al. 1990). The role of various redox sensors that detect and respond to oxidants specifically H_2O_2 is also important in this content. In this context the induction of a gene encoding a potential hybrid His-kinase is of particular interest. His-kinases and two-component signal transduction systems are well represented in *Arabidopsis* genome and have already been shown to modulate cellular responses to other growth regulators. H_2O_2-induced expression of His-kinase suggests strangely that it may function as H_2O_2 sensor in plants mediated with other downstream MAPK systems.

8.4.4 ROS: Part of Signaling Network Involving Kinases and Phosphatases

In order to exert such a profound effect on plant metabolism, ROS must utilize and/or interfere with other signaling molecules (Mittler et al. 2011). In other words, ROS signaling is highly integrated with other hormonal signaling networks that help the system in processing and transmitting environmental inputs to evoke appropriate responses under environmental constraints. Involvement of almost all recognized plant growth regulators like GAs, ABA, IAA, SA, and C_2H_4 together with ROS signaling (both feed forward and backward) allows the plant to regulate short-term acclimation, long-term adaptive responses, and developmental processes (Fig. 8.6). The protective signaling events regulated by plant hormones which may lead to activation of acclimation responses, such as reduction of stomatal conductance and regulation of hydraulic conductivity, found to be largely mediated by ROS (Miller et al. 2010; Sakomoto et al. 2008). There exists cross talk between ROS and hormones like GAs, ABA, IAA, SA, and C_2H_4, which ultimately confer defense, stress acclimation, cell death response, and developmental cues (Mittler et al. 2011; Miller et al. 2010).

H_2O_2 was found to induce accumulation of stress hormones such as C_2H_4 and salicylic acid (Leon et al. 1995). Plant hormones are not only downstream of ROS signal; ROS themselves are secondary messengers in many hormone-mediated signaling pathways (Chen et al. 1993a, b).

Therefore it is conceivable that feedback/feed forward interactions between ROS and hormones occur (Fig. 8.6).

ABA is implicated in a number of abiotic stress responses associated with dehydration, such as drought, cold, salinity, and heat shock. One of the physiological ABA responses is stomatal closure that prevents excessive transpiration. As a consequence, availability of CO_2 for fixation of the C_3 cycle is limited, which may, in turn, enhance ROS production in the chloroplast. Despite this well-known caused link between ABA and ROS, little attention has been drawn on the relationship between these two signaling molecules and their pathways. Earlier studies have shown that ABA can modulate activities of antioxidant enzymes and can affect expression of genes encoding them (Gong et al. 1998; Kurepa et al. 1997). Both H_2O_2 and ABA independently acclimate maize seedlings to chilling; however, the relative position of the messengers in this process is not yet clear. The antioxidant gene Cat1 induction takes place independently both by ABA and ROS. Because H_2O_2 levels rapidly increase upon ABA treatment, H_2O_2 was proposed to be a downstream component of ABA signaling involving Cat1 induction (Guan et al. 2000). Therefore, a model for signaling pathway leading to downstream ABA responses starts to emerge and is in part based on the studies of regulation of stomatal conductance by ABA. Briefly, stomatal closure is mediated via a reduction of osmolyte concentrations (both inorganic and organic) in the guard cells. ABA initiates the process via an increase in cytosolic Ca^{2+} level that can be released from internal stores via ADP-ribose and from external sources via influx across plasma membrane. ABA signaling pathway is negatively regulated by PP2C-like phosphatases that are transcriptionally activated by ABA, creating a negative feedback loop (Gosti et al. 1993). It was demonstrated that ABA induces generation of H_2O_2 in stomatal guard cells and H_2O_2-activated Ca^{2+} influx as well as stomatal closure. Alternatively, H_2O_2 may modulate ABA responses by compromising the negative effect of PP2Cs on ABA pathway, because a tobacco homologue of PP2Cs, implicated in ABA signaling, is transcriptionally downregulated by a number of oxidative stress stimuli (Varnová et al. 2002). Thus, the condition where ROS enhance and/or prolong downstream ABA responses or activate the pathway by decreasing the negative regulator PP2C may be envisaged.

Various oxidants, especially H_2O_2, cause Ca^{2+} influx into cytoplasm and consequently in mitochondria and nucleus. In fact, Ca^{2+} can be used as a signal molecule to respond oxidant stimuli which can activate and repress proteins and gene transcription. Generally, oxidants activate Ca^{2+} channels, repress pumps, and can even reverse Na^+/Ca^{2+} exchangers (Cunninghum and Fink 1996). However, the mechanisms by which ROS exerts such regulation are poorly understood.

Calcineurin is one of the least characterized Ca^{2+}-dependent phosphates. It is calcium-/calmodulin-activated serine/threonine phosphatase (PP2B) which is an important enzyme in Ca^{2+}-dependent eukaryotic signal transduction pathways. Calcineurin plays a critical role in cellular responses to various extracellular signals and environmental stresses (Asai et al. 1999; Krebs 1997). In plants calcineurin mediates stress adaptation (Asai et al. 1999). Calcineurin activity is found to be extremely sensitive to cellular redox state (Krebs 1997). H_2O_2 can completely abrogate calcineurin-mediated NF-AT transactivation in response to stimulation (Asai et al. 1999). It has also been shown that calcineurin inhibitors protect against Ca^{2+}-induced cell toxicity, which is observed when cells are transferred from media without Ca^{2+} to media containing physiological concentrations of Ca^{2+}. These observations link calcineurin with both oxidative stress and calcium signaling. The mechanism by which calcineurin mediates interactions of oxidative stress with Ca^{2+} signaling is not yet understood. It might involve Ca^{2+} transport through $InsP_3$-sensitive channel in ER/SR. Activation of calcineurin by Ca^{2+} can cause further Ca^{2+} influx into the cytoplasm. Calcineurin transduction pathways are well characterized in yeast, where enzyme promotes growth in high Ca^{2+} environments by dephosphorylation of TcnIp/CrzIp transcription factor. Oxidative stress causes rising Ca^{2+}

concentrations in the cytoplasm and the nucleus. Ca^{2+} in turn activates CaM binding to calcineurin and enhances phosphatase activity.

Ethylene, another stress hormone, also found to have interaction with ROS in their signaling network. Tobacco plants with reduced peroxisomal catalase activity produce C_2H_4 as an earliest response of high light irradiance (Chamnongpol et al. 1998). Exogenous application of H_2O_2 increases C_2H_4 production in pine needles in a concentration-dependent manner (Ivenish and Tillberg 1995). Ozone, which is known to form ROS in apoplast, induces accumulation of C_2H_4 in tobacco plants. C_2H_4 in all cases seems to originate from de novo synthesis, as ACC (precursor of C_2H_4 biosynthesis) increases concomitantly with C_2H_4 production (Ivenish and Tillberg 1995). Recently, O_3-induced O_2^- accumulation and cell death have been demonstrated to be substantially reduced in ethylene-insensitive *Arabidopsis* mutant ein-2, whereas ethylene-insensitive *Arabidopsis* mutant eto-1 is hypersensitive to ozone. This strongly suggests that C_2H_4 has a potentiating role in oxidative cell death by controlling O_2^- accumulation.

Protein kinases and phosphatases are vital for transduction ROS signals. A cascade of three protein kinases mitogen-activated protein kinase kinase kinase (MAPKKK), protein kinase kinase (MAPKK), and protein kinase (MAPK) is a conserved functional module in a variety of signal transduction pathways in diverse organisms (Hirt 2000). A MAPK module that senses H_2O_2 signal and translate it to the expression of defense genes (GST 6, HSP 18.2) was identified in *Arabidopsis* (Kovtun et al. 2000). This module consists of an upstream kinase AMP 1 and downstream kinases AtMpk 3 and AtMPK 6 H_2O_2, but not auxins; ABA activates this kinase cascade (Kovtun et al. 2000).

Therefore, plant hormones are not only located downstream of ROS signal, but ROS themselves are also secondary messengers in many hormone signaling pathways. Ca^{2+} can also be exploited as a signaling intermediate under oxidative stress. So, both feedback and feed forward interactions may conceivably occur between different endogenous growth regulators, Ca^{2+} and ROS.

8.5 Conclusion and Perspective

It is now clear that ROS is involved in acclamatory stress tolerance in plants. Because ROS are linked to acclamatory stress responses, deciphering ROS signaling is likely to have a significant impact on agriculture and biotechnology which could lead to the development of crops with enhanced yield under suboptimal conditions. ROS through redox-sensing device interact with other major signaling pathways, to trigger complex metabolism leading to the regulation of expression of stress inducible genes. The complexity of plant responses to multiple stresses and ROS network has shown a need to develop new research approach to elucidate the overwhelming benefit of ROS in stress acclimation and defense mechanism, which ultimately will enable us to harvest the yield potential of crops. The management of oxidative stress and the ROS-mediated upregulation of specific stress inducible genes need to be evaluated in important agricultural crops so that we can adopt the positive ROS network pathways as a highly beneficial prerequisite for stress acclimation and defense.

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9 Cyclic Nucleotide-Gated Channels: Essential Signaling Components in Plants for Fertilization and Immunity Responses

Reiaz Ul Rehman

Abstract

The Cyclic Nucleotide-Gated Channels (CNGCs) in plants are responsible for conducting mono and divalent cations such as Ca^{2+}, Pb^{2+}, Na^{+}, and K^{+}. The CNGCs have been identified in different plant species, namely, *Arabidopsis thaliana* where 20 have been genetically identified. They express during different developmental stages of the plant organ, tissue, or cell indicating the specificity in their function. The CNGCs have been implicated in diverse responses in plants from stress tolerance (both biotic and abiotic) to transpiration and fertilization. These responses in plants are due to the presence of complex signaling network of which CNGCs are a part. Thus far CNGCs were implicated in the hormone-mediated pathways (GA, IAA, ABA, JA) leading to developmental responses, NO-mediated pathways leading to plant defense and immunity responses, and cyclic nucleotides monophosphates (cNMPs) and Calmodulin-mediated pathways leading to regulation of cation conduction into the cell. The cNMPs and calcium acts as the secondary messenger in these signaling pathways transducing the signal from the environment into the cell.

Keywords

Cyclic Nucleotide-Gated Channels (CNGCs) • Calmodulin (CaM) • CaM-binding domain (CaMBD) • Cyclic nucleotide monophosphates (cNMPs) • "P-loop" channels

9.1 Introduction

The plant CNGCs belong to the family of cation conducting channels which were first identified in 1998 (Schuurink et al. 1998). The family comprises of nonselective, monovalent and divalent cation conducting channels (Kaplan et al. 2007; Köhler et al. 1999; Ali et al. 2006; Gobert et al. 2006), and till date, 56 coding sequences of these

R. Ul Rehman (✉)
Department of Bioresources, University of Kashmir, Srinagar 190006, Jammu and Kashmir, India
e-mail: reiazrehman@yahoo.co.in

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Table 9.1 Identified coding sequences of cation-conducting channels

Arabidopsis thaliana CNGCs	Accession No.	References	Probable function
AtCNGC1	AT5G53130	Sunkar et al. (2000) and Ma et al. (2006)	Metal tolerance
AtCNGC2	AT5G15410	Chan et al. (2003), Chaiwongsar et al. (2009), and Clough et al. (2000)	Plant development (dwarfism), fertility Resistance to pathogens
AtCNGC3	AT2G46430	Gobert et al. (2006)	Ion homeostasis
AtCNGC4	AT5G54250	Mäser et al. (2001), Moeder et al. (2011), Balague et al. (2003), and Jurkowski et al. (2004)	Slow growth and low fertility plant immunity
AtCNGC5	AT5G57940		
AtCNGC6	AT2G23980		
AtCNGC7	AT1G15990	Tunc-Ozdemir et al. (2013) and Bock et al. (2006)	Male gametophyte development
AtCNGC8	AT1G19780	Tunc-Ozdemir et al. (2013) and Bock et al. (2006)	Male gametophyte development
AtCNGC9	AT4G30560	Tunc-Ozdemir et al. (2013)	Male gametophyte development
AtCNGC10	AT1G01340	Tunc-Ozdemir et al. (2013), Guo et al. (2010), Borsics et al. (2007), Li et al. (2005), and Christopher et al. (2007)	Male gametophyte development Transports Ca^{2+}, Mg^{2+} lower K^{+} levels starch accumulation Root gravitropic and cell division and growth
AtCNGC11	AT2G46440	Moeder et al. (2011), Urquhart et al. (2011), Yoshioka et al. (2006), and Urquhart et al. (2011)	Plant defense Synergistic roles in senescence
AtCNGC12	AT2G46450	Moeder et al. (2011), Urquhart et al. (2011), Yoshioka et al. (2006), and Urquhart et al. (2011)	Plant defense Synergistic roles in senescence
AtCNGC13	AT4G01010		
AtCNGC14	AT2G24610		
AtCNGC15	AT2G28260		
AtCNGC16	AT3G48010	Tunc-Ozdemir et al. (2013) and Bock et al. (2006)	Male gametophyte development
AtCNGC17	AT4G30360		
AtCNGC18	AT5G14870	Tunc-Ozdemir et al. (2013), Frietsch et al. (2007), and Bock et al. (2006)	Male gametophyte development
AtCNGC19	AT3G17690	Mosher et al. (2010), Kugler et al. (2009), and Urquhart (2010)	Salt stress responses Pathogen response
AtCNGC20	AT3G17700	Kugler et al. (2009) and Urquhart (2010)	Salt stress responses Pathogen response

All CNGCs are PM localized; however, Zelman et al. (2012) proposes on basis of BLAST analysis that CNGC 20 may be chloroplast localized. Based on *Arabidopsis* MIPS database, CNGCs contain putative signal peptide sequences targeting to membrane, chloroplast, mitochondria, and secretory pathway (Reddy et al. 2002)

channels have been identified in the genome of *Arabidopsis thaliana*. Out of these 56 coding sequences of channels, 20 have been identified as CNGCs Table 9.1 (Ward et al. 2009). The functional characterization of CNGCs has been done either by their expression in heterologous systems or by the knockouts of particular CNGC to study the mutant phenotypes (Talke et al. 2003; Ma et al. 2010). The CNGCs are present in many monocot and dicot plant species besides *Arabidopsis thali-*

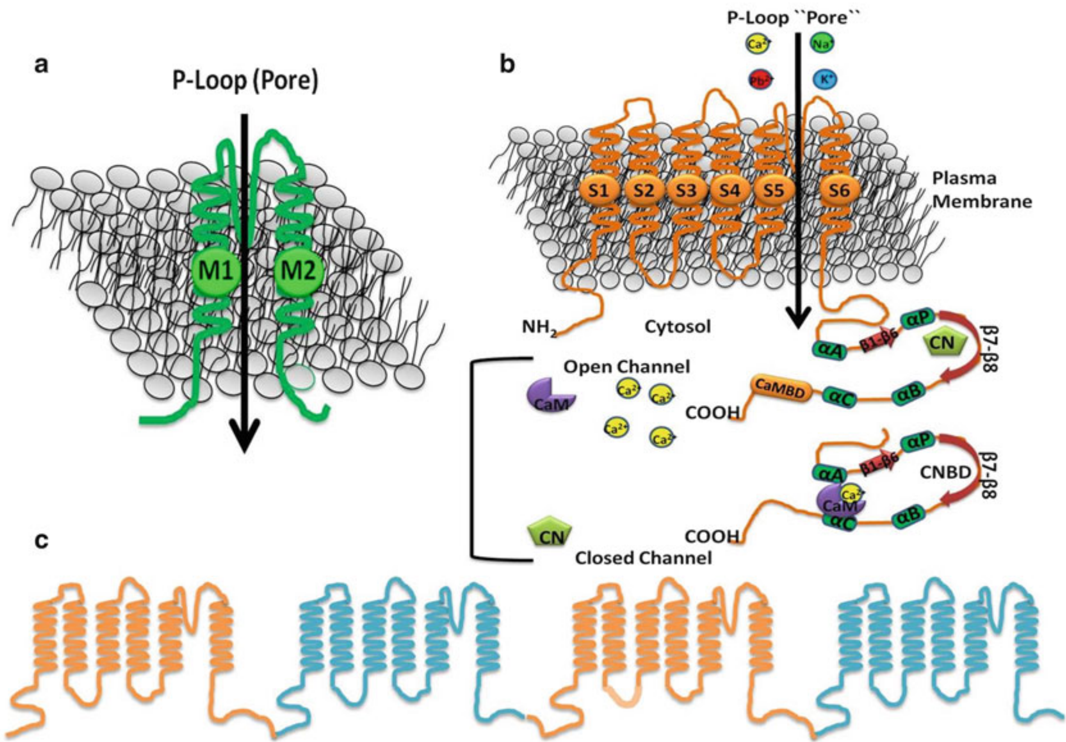

Fig. 9.1 (**a**) Bacterial transmembrane channel with two coils one P-pore. (**b**) Working model for plant Cyclic Nucleotide-Gated Channels. When CN binds to the CNBD of a CNGC, it opens the channel, and cations, such as Na^+, K^+, Ca^{2+} and Pb^{2+}, can pass through the pore subjected to a selectivity filter (between S5 and S6). Following increase in Ca^{2+} levels in the cytosol, Ca^{2+} binds to CaM, and the Ca^{2+}-CaM complex binds to the CaMBS, presumably preventing binding of CNs to the channel, leading to its closure (After Kaplan et al. 2007). (**c**) A representation of the secondary structure of an animal Ca^{2+} channel has revealed that it is formed of one large polypeptide of four repeats with each repeat having six transmembrane/one pore structure

ana. The screen for calmodulin (CaM)-binding proteins in barley (*Hordeum vulgare*) led to identification of HvCBT1 (CaM-binding transporter) in the aleurone plasma membranes (Schuurink et al. 1998). In tobacco (*Nicotiana tobaccum*), two sequences NtCBP4 and NtCBP7 homologous to HvCBT1 were identified (Arazi et al. 1999). Further, the GenBank Blast queries revealed CNGC like sequences in *Oryza sativa*, *Phaseolus vulgaris,* and other plant species (Altschul et al. 1997). The presence of many CNGCs in plant species belonging to same phylogenetic subgroups points to their involvement in similar physiological functions (Talke et al. 2003). The predicted structure of plant CNGCs has revealed 6 transmembrane domains (S1–S6), a pore domain (between S5 and S6), and the *C*-terminal CNB (cyclic nucleotide binding) domain and CaMB (calmodulin-binding) domain (Fig. 9.1) (Demidchik et al. 2002; Véry and Sentenac 2002; White et al. 2002), the CN-binding domain (CNBD) that partly overlaps with a CaM-binding site (CaMBS) (Köhler et al. 1999; Arazi et al. 2000; Köhler and Neuhaus 2000). Although plant CNGCs show similarities in amino acid sequence and overall structure to the family of six membrane-spanning domains K^+-selective shaker family channels and to animal nonselective cation CNGCs, they differ in several key aspects. Firstly, in animal CNGCs, the location of the CaMB region has been found to

be at the *N*-termini (Liu et al. 1994; Grunwald et al. 1998). Secondly, the pore sequence of plant CNGCs does not contain the GYGD K^+-selectivity filter sequence nor does it contain the sequence of the animal CNGC selectivity filter. Thus, the plant CNGC selectivity filter has unique properties in sequence and in function (Hua et al. 2003a). Therefore, plant CNGCs may differ from their animal counterparts in selectivity towards K^+, Na^+, Ca^{2+}, and possibly towards other cations (Kaplan et al. 2007).

The plants CNGCs are the members of the superfamily "P-loop" of cation channels which are present in all prokaryotes and eukaryotes (Ward et al. 2009). Given that the "P-loop" channels are present in all prokaryotes and eukaryotes which are capable of conducting the range of cations, it could be said that their evolution took place very early for fulfilling the cellular functions (Zhorov and Tikhonov 2004). The bacterial (prokaryotic) cation channel polypeptide is comprised of two helices (M1 and M2) surrounding the membrane P-loop (pore loop). While as there are six transmembrane (TM) regions (S1–S6) formed by the polypeptide in animals and the plants (Eukaryotes), in case of the plants, the "P-loop" is present between the S5 and S6 region on the plasma membrane (Hua et al. 2003b). The channels have the *N*-terminal (amino) and *C*-terminal (Carboxy) groups on the cytosolic side of the membrane. The "P-Loop" is highly conserved with a α helix, a turn, and a random coil (Zhorov and Tikhonov 2004). The "P-loop" is oriented in such a way on the membrane so as to form an ion-conducting pathway, and it contains certain amino acids which form the ion selective filter (Ward et al. 2009). The quaternary structure in animals has revealed that it is formed of four repeat sections with each section containing six transmembrane P-loop structure (Zhorov and Tikhonov 2004). The mammalian CNGCs are heterotetrameric (Kaupp and Seifert 2002), and it was found that bovine CNGC was composed of three CNGA1 and one CNGB1 subunits (Zhong et al. 2002). The genes encoding the Ca^{2+} conducting four repeat P-loop channels are absent in plants (Zelman et al. 2012).

The eukaryotic channels with six transmembrane P-loop structure are common to both ligand-gated and the voltage-gated superfamilies of channels (Mäser et al. 2001). The voltage-gated channel configuration changes lead to the opening or closing of these channels in plants as well as animals. The configuration change is affected by the membrane potential (E_m), and the closing of the channel leads to the blocking of conductance (Zelman et al. 2012). The positively charged amino acids in the S4 domain act as the voltage sensors which respond to the E_m by altering the gating of the pore (Hua et al. 2003b). The conductance through the pore can also take place by the ligand gating which takes place by binding of ligands at the *N*- or *C*-terminals present in the cytosol (Biel 2009). The CNGCs in animals are gated allosterically by cyclic nucleotides (cAMP and or cGMP) and calmodulin (CaM). The binding of CaM in the presence of Ca^{2+} to CaM-binding domain (CaMBD) results in the closing of the channel. CaM binding in animals takes place at the *N*-terminal while in plants the CaM binding takes place at the *C*-terminal (Kaplan et al. 2007; Zelman et al. 2012). Furthermore, the CNs can bind CNGCs on *C*-terminal. In animal cells there are two types of channels whose conductance is regulated by CNs (Biel 2009). One class of the channels binds directly to CNs, and the changes in E_m have no effect on these. However, the second class of channels is dependent on $E_{m,}$ i.e., they are hyperpolarization activated and they bind to cyclic nucleotides. The binding to CNs is said to increase the probability of open channels at the particular E_m (Zelman et al. 2012). The plant channels are also reported to posses the hyperpolarization-activated voltage-gated channels (Hua et al. 2003b), but this operating mode is not yet clear (Zelman et al. 2012). Additionally, the plants and animals CNGCs contain cyclic nucleotide-binding domains (CNBDs), but these CNBDs are absent from the unicellular fungi (Talke et al. 2003). Some structural aspects of plant CNBDs are similar to that of bacterial and animal analogs. It has been shown that in the plants, CNBDs overlaps with the CaMBDs close to the C-termini (Hua et al. 2003a). In the bacterial CNGCs, the CaMBD sequence is absent

(Cukkemane et al. 2011). However, in the animals, the CaMBD is located near the *N*-terminal (Ungerer et al. 2011). CaM binds to CNGCs in both animals and plant CNGCs to regulate conductance (Kaplan et al. 2007; Zelman et al. 2012). The CNBDs in plants have also been shown to contain GAF domains which also bind to the cyclic nucleotides. These GAFs were identified in bacteria, yeast, humans, *A. thaliana,* and cyanobacteria (Aravind and Ponting 1997). GAF domain can bind to cAMP, cGMP, and cNMP and other small molecules (Bridges et al. 2005). The CNBD of plant CNGCs are also present in K^+ selective channels families called as "Shake like." The effect of CN on these K^+ selective channels is different than that on CNGCs (Gaynard et al. 1996). The cNMPs elevation in the cytosol reduces the conductance in K^+ selective channels having CNBDs (Zelman et al. 2012), while in plants the CNGCs channel elevation of cNMPs activates these channels and increases the conductance (Leng et al. 1999, 2002; Lemtiri-Chlieh and Berkowitz 2004; Ali et al. 2007).

9.1.1 Interaction of Ca^{2+} and CNGCs

Ca^{2+} is a pivotal secondary messenger in plant cells and has a role in signaling cascade. The transport of Ca^{2+} across the membrane is regulated during resting and signaling events which are in term specified by particular physiological response (McAinsh and Pittman 2009). In plant cells Ca^{2+} is transported by channels belonging to many gene families. These channels are of various types such as Ca^{2+} permeable, P-type-ATPases, and the H^+ antiporters. Among these CNGCs belong to the Ca^{2+} permeable ion channels. These are nonselective cation channels which have been characterized by various electrophysiological and radio tracer methods (Demidchik and Maathuis 2007; Roy et al. 2008). The expression studies have revealed that there is alteration in ion fluxes into tissues as well as their accumulation in the tissues (Ali et al. 2007; Guo et al. 2008). The heterologous expression of some of the CNGCs have helped in detecting Ca^{2+}, Na^+, K^+ permeable ion channels. Further, the mutants of certain members of CNGCs have revealed the Ca^{2+}-related phenotypes (Hampton et al. 2005; Chin et al. 2009). The mutant lines of CNGC1 were reported to have reduction in Ca^{2+} uptake, while the mutant lines of CNGC2 were shown to be hypersensitive towards the Ca^{2+} in the growth medium (Chan et al. 2003; Ma et al. 2006). Upon mining of the PiiMS database, it was revealed that two independent mutations of AtCNGC2 showed an increase in shoot calcium (Ca^{2+}) and decrease in shoot potassium (K^+) as compared to the wild types. However, the type of cell in which this increased Ca^{2+} is stored is not clear and it remains to be established if the poor growth phenotype is due to the defective Ca^{2+} signaling or due to the defective storage (Chan et al. 2008).

9.1.2 cNMPs in Plants

The signal transduction is important for plants in their development, control of metabolism, uptake of nutrients, and responses to environment. The external stimuli lead to the changes in concentration of second messengers within the cells. Among the secondary messengers present in plants are the cyclic nucleotide monophosphates (cNMPs, cAMP, and cGMP) (Newton and Smith 2004). The cyclic nucleotide monophosphates were initially discovered in animals. They have been widely studied in animals and some prokaryotes, and their role in signaling has been established (Donaldson et al. 2004; Newton and Smith 2004). The enzymes responsible for synthesis of cNMPs are adenyl/guanyl cyclases (Moutinho et al. 2001; Ludidi and Gehring 2003). Besides some specific phosphodiesterases responsible for the cNMPs degradation have also been reported (Newton and Smith 2004). The cNMPs have been found in various parts of plants and are known to regulate many processes, such as the opening of stomata (Newton and Smith 2004), development of chloroplasts (Bowler et al. 1994), functioning of GA (Penson et al. 1996), flux of cations (Maathuis and Sanders 2001; Essah et al. 2003; Maathuis 2006; Rubio et al. 2007), and response to pathogens (Durner et al.

Table 9.2 Functions of plant cNMPs in plants

Cyclic nucleotide monophosphates (cNMPs)	Plant response	References
cGMP	Hormone-induced signals (protein phosphorylation)	Isner et al. (2012)
	Protection against salt stress	Maathuis and Sanders (2001) and Maathuis (2006)
	NO-induced signals	Prado et al. (2004)
	Protection against salt and osmotic stress	Donaldson et al. (2004)
	Phytochrome signaling	Wu et al. (1996)
	Indole acetic acid-induced adventitious rooting	Pagnussat et al. (2003)
	Gibberellic acid-induced gene expression	Penson et al. (1996)
cAMP	Pollen tube growth and reorientation	Moutinho et al. (2001)
	Fertilization	Tsuruhara and Tezuka (2001)
	Cell division (mitosis)	Ehsan et al. (1998)
	Pathogen response (hypersensitive response to fungal elicitors)	Cooke et al. (1994)
	NO-mediated activation for defense gene induction	Delledonne et al. (1998)

1998; Ma et al. 2009). However, it was recently reported that besides gibberellic acid (GA), other plant hormones such as Indoleacetic acid (IAA), abscisic acid (ABA), and jasmonic acid (JA) also evoked the cGMP changes in the cytoplasm (Isner et al. 2012). The cGMP has been suggested to have a role in phytochrome signal transduction in anthocyanin biosynthesis pathway (Barnes et al. 1995). This cGMP pathway was antagonistic to phytochrome signaling mediated by calcium/calmodulin pathway, and the genes encoding the enzymes in these pathways are attenuated by cGMP and Ca^{2+} (Wu et al. 1996). Furthermore, the microarray analysis has revealed that approximately 1,000 genes responded upon the treatment of cGMP indicating the modulation of transcription by cGMP (Maathuis 2006). A recent study has revealed that the hormone-induced cGMP signaling takes place by protein phosphorylation. The phosphoproteomic analysis in *Arabidopsis thaliana* root microsomal protein showed that upon treatment of cGMP, 14 proteins were phosphorylated within minutes (Isner et al. 2012). The role of cNMPs in phytochrome signaling was reported in *Cyanobacterium* (Ohmori and Okamoto 2004) and photo transduction in retina of vertebrates. The above reports point towards the evolutionary conservation of cNMPs among the different organisms (Kaplan et al. 2007). Table 9.2 depicts some of the functions of plant cNMPs in plants.

9.2 Immune Responses of Plants

In nature plants are exposed to various stresses and the biotic stress is one of them which pose serious challenges. The biotic stresses are caused by diversity of disease causing infectious microorganisms. Only certain microorganisms can cause infections because plants possess various preformed defense systems and the induced defense responses. The initial line of induced defense (immune system) in plants is called as Microbe-associated Molecular Pattern, MAMPs, and is activated by conserved molecules among the pathogens (Zhang and Zhou 2010). The pathogens on the other hand have been reported to have evolved the effector proteins against this initial response by the plants (Bent and Mackey 2007). Due to the presence of these arsenals by the pathogens, the plants are believed to have evolved the second line of defense which is even stronger and is mediated by the expression of

resistance (R) genes. The products of R genes have a role in interacting or monitoring the effectors from the pathogens (Bent and Mackey 2007). The resistance impacted by the R gene is responsible for developing the hypersensitive response (HR) which is evident by the apoptosis-like cell death in and around the point of entry of the pathogen (Heath 2000). The HR response is not necessarily required always for the pathogen resistance, but it is followed by the salicylic acid (SA) increase and the pathogenesis-related (PR) genes induction (Clough et al. 2000; Jurkowski et al. 2004; Vlot et al. 2008). The recognition of pathogen leads to other defense response in the form of a reactive oxygen species (ROS) production (Torres 2010). Among the early responses are also the changes in ion fluxes which include influx of ions such as H^+ and Ca^{2+} and efflux of ions such as K^+ and Cl^- (Atkinson et al. 1996). Among the ions Ca^{2+} is considered as the second messenger responsible for the induction of defense responses in plants (Moeder et al. 2011). This is thought to be due to its elevation upon the pathogen attack or the treatment of elicitors. The Ca^{2+} ions are also reported to activate oxidative burst upon the treatment with elicitors (Sasabe et al. 2000). They Ca^{2+} ions are also reported to activate the induction (pathogen/elicitor-induced) process of cell death (Atkinson et al. 1996; Levine et al. 1996; Xu and Heath 1998; Sasabe et al. 2000). The role of Ca^{2+} ions has also been thought to be in SA-induced PR gene expression (Doke et al. 1996). Moreover, there are other pathways which are controlled by ethylene (ET) and Jasmonic acid (JA) (Pieterse and van Loon 1999; Glazebrook 2005), and there is a constant cross talk among the SA and ET/JA hormonal signaling pathways which are generally thought to be antagonistic (Glazebrook 2005). Therefore, the choice of plants among the either signaling pathways would depend on the type of pathogen attacking it (Glazebrook 2005; Spoel et al. 2007). It is being said with certainty that the Ca^{2+} ion fluxes have the role in plant defense response activations, and among these responses, ROS induction and MAPK (mitogen-activated protein kinases) activation have been ascribed to Ca^{2+} ion influx (Romeis et al. 1999; Kurusu et al. 2005; Ogasawara et al. 2008). There is not much information about the identity of the channels that generate the Ca^{2+} ion fluxes after the pathogen recognition (Moeder et al. 2011).

9.2.1 CNGCs in Plant Immunity

It has been reported in *Arabidopsis* that there are more than 150 cation transport proteins (Mäser et al. 2001), and among these are the TPC1 (two-pore channel), GLRs (ionotrophic glutamate receptors), and CNGCs (cyclic nucleotide-gated ion channels) (Kurusu et al. 2005; Demidchik and Maathuis 2007; Dietrich et al. 2010; Ali et al. 2007; Urquhart et al. 2007). So far, among the channels, only CNGCs have emerged as the strong candidate for Ca^{2+} conduction in plant immune responses (Moeder et al. 2011; Talke et al. 2003; Kaplan et al. 2007). Out of the 20 family members of *Arabidopsis thaliana* CNGCs, only four have been identified through mutant screen to be involved in immunity responses.

The first one to be identified was dnd 1 (defense, no death 1) so named because of the phenotype which maintains the R-gene-mediated resistance and reduce HR cell death (Yu et al. 1998). The dnd 1 mutant was later identified as the null mutant of AtCNGC2 (Clough et al. 2000). The dnd 1 mutant plants also exhibit the elevation in SA, constitutive expression of PR genes, and the increase in resistance to *Pseudomonas syringae* and *Hyaloperonospora arabidopsidis* (Yu et al. 1998; Genger et al. 2008). The second and the third mutant which were identified are hlm 1 (HR-lesion mimic 1) and dnd 2 (defense no death 2). These two were identified as the null mutants of the AtCNGC4 (Yu et al. 2000; Balague et al. 2003; Jurkowski et al. 2004). Further these mutants were shown to exhibit the similar phenotypes to dnd 1 (Balague et al. 2003; Jurkowski et al. 2004). It was also shown that the barley mutant nec 1 displayed some features of *dnd1, hlm 1,* and *dnd 2* and had mutation in homolog of AtCNGC4 (Rostoks et al. 2006). The double knock out mutants of the AtCNGC2 and AtCNGC4 were reported to show the additional phenotypic effects than the above (Mäser et al. 2001; Jurkowski et al. 2004).

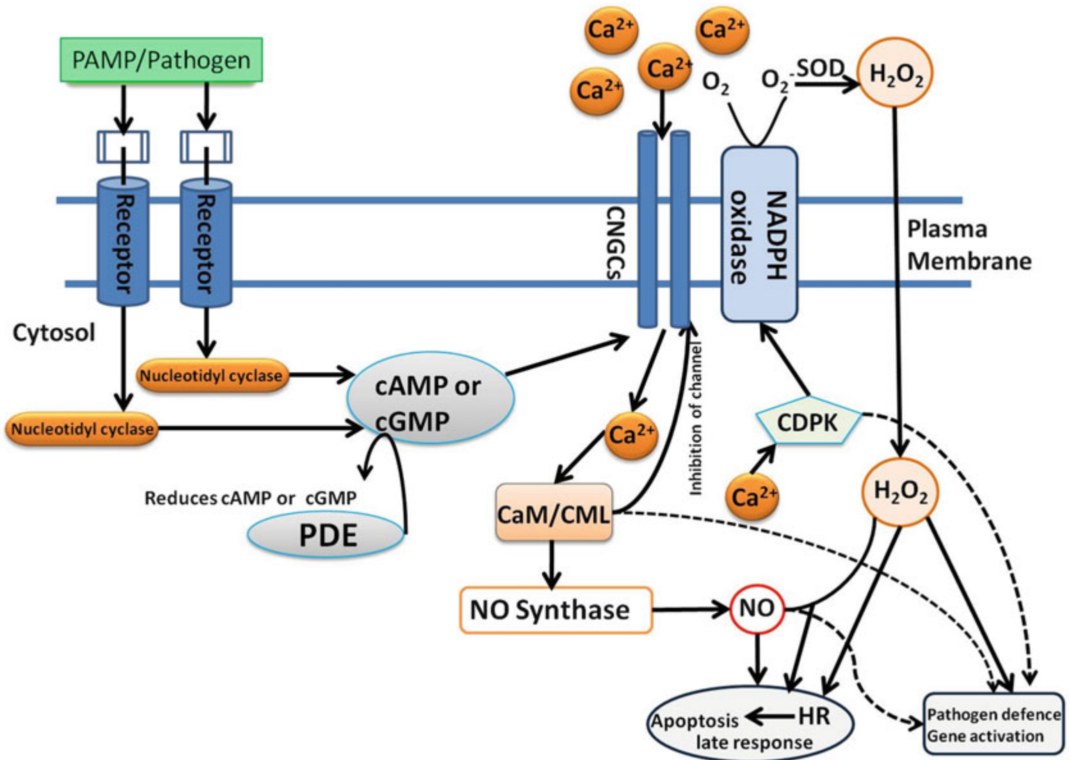

Fig. 9.2 Model of possible mechanisms in the plant immune response signal transduction pathway. Pathogen recognition by a receptor leads to the activation of nucleotidyl cyclase (NC; localized on the plasma membrane or in the cytosol) and a rise in the concentration of cyclic nucleotide. The increase in the cyclic nucleotide concentration results in the activation of the cyclic nucleotide-gated ion channel (*CNGC*) and cytosolic Ca^{2+} elevation. Ca^{2+} elevation increases the amount of Ca^{2+} bound to calmodulin (*CaM*) (or CaM-like protein (*CML*)). Ca^{2+}/CaM regulates the synthesis of downstream signaling components (nitric oxide (*NO*) and hydrogen peroxide (H_2O_2)), which are essential for the development of the hypersensitive response (*HR*). The increased amounts of Ca^{2+}/CaM in the cytosol also compete with cyclic nucleotide for binding to the CNGC, blocking further Ca^{2+} conductance by the channel. The increase in Ca^{2+} elevation also results in the binding of Ca^{2+}-dependent protein kinase (*CDPK*) which can activate H_2O_2 production through regulation of NADPH oxidase activity. CaM, CDPK, NO, and H_2O_2 also play pivotal roles in activating the expression of pathogen defense genes. In all cases, *arrows* indicate activation unless noted otherwise. For clarity, some *arrows* are shown with *broken lines*. *PAMP* pathogen-associated molecular pattern, *PDE* phosphodiesterase, *SOD* superoxide dismutase

Subsequently, the fourth mutant which was identified was cpr22 (constitutive expressor of PR genes22), a gain-of-function mutant (Yoshioka et al. 2001, 2006). The phenotypes in this mutant are also similar to dnd1 and dnd2/hlm1 (Yoshioka et al. 2001). However, the induction of HR upon infection with *Pseudomonas syringae* pv. Tomato distinguishes it from the dnd1 and dnd2/hlm1 (Yoshioka et al. 2006). The mutation cpr22 was identified as a 3-kb deletion generating a chimeric gene AtCNGC11/12 (Yoshioka et al. 2006; Baxter et al. 2008). The cpr22 mutants showed lesion formation and the T-DNA insertion knockout mutants of AtCNGC11 and AtCNGC12 acted as the positive regulators of R gene mediated resistance responses (Moeder et al. 2011; Yoshioka et al. 2006). Fig.9.2

9.3 CNGCs in Fertilization

9.3.1 Role of Ca^{2+} in Pollen

Calcium is an essentially important signaling element which performs various physiological and regulatory roles during fertilization processes

in flowering plants. The calcium elevation is an important indicator of the fertility in plants (Ge et al. 2007). The calcium exists in three forms in plants, viz., covalently bound, loosely bound forming ionic bonding with anions, and the free cytosolic form. The free cytosolic calcium acts as an important secondary messenger in signaling processes. In flowering plants the calcium is required by the pollen for germination, elongation, and reorientation.

There are large numbers of evidences which reveal that cytoplasmic streaming is controlled by Ca^{2+}. The streaming takes place normally at basal levels, but when the Ca^{2+} concentration elevates to 1 µM or higher, it takes place rapidly and is reversibly inhibited (Taylor and Hepler 1997). At higher levels of Ca^{2+}, the F-actin fragmentation takes place (Yokota et al. 1998) that is the reason for non-visibility of thick microfilaments in tube apex. The actin-binding proteins such as villin are responsible for the inhibition and fragmentation activities (Vidali et al. 2001). Furthermore the tube apex $[Ca^{2+}]_c$ levels are also higher to the extent that it can inhibit streaming which is brought about by inhibiting myosin motor activity. Therefore this activity of apical $[Ca^{2+}]_c$ controlling the motion by slowing or stopping the actin may consequently help in regulating vesicle docking and fusion at the plasma membrane. These activities taking place at the tube apex have a very intimate relationship with reorientation process in the pollen tube. This has been demonstrated that by altering $[Ca^{2+}]_c$ at the tube apex, results in change of growth axis towards the zone containing high $[Ca^{2+}]_c$ (Malhó and Trewavas 1996). The growth of the pollen tube depends primarily on polarized exocytosis at growing tips, and Ca^{2+} is implicated in this process (Malhó et al. 2000). When the fluorescent dye FM 1–43 was used for measuring of endo-exocytosis in growing pollen tubes, the fluorescence hot spot was visible at the apex (Camacho and Malhó 2003) and eventually upon tube reorientation the fluorescence hot spot changed at the dome to which cell bending took place. These observations led to suggestions that vesicle relocates and the distribution of fusion events are asymmetric. The released cargo upon vesicle fusion is required for new cell wall construction and growth which includes elongation of the existing wall (Malhó and Trewavas 1996). These results of $[Ca^{2+}]_c$ mapping suggests the regulatory role in coupling in-between growth and endo-exocytosis. Furthermore, work of Camacho and Malhó (2003) suggests that cell growth process is not fully dependent on Ca^{2+}-mediated stimulation of exocytosis. Other mechanisms to regulate membrane recycling and wall strength must be present. The conformity to above results is provided by Roy et al. (1999) who used the Yariv reagent, an inhibitor of growth but has no effect on secretion and thus suggesting that the role of Ca^{2+}-dependent exocytosis is mainly for secreting the cell wall components, and it works independently of cell elongation. This explains why rise in levels of apical Ca^{2+} only resulted in increase in secretion and growth axis reorientation but not high growth rates. The mechanisms regulating $[Ca^{2+}]_c$ at pollen tube apex are still controversial. Holdaway-Clarke et al. (1997) did a study where they combined measurements of $[Ca^{2+}]_c$ and Ca^{2+} fluxes to find apparent discrepancies between internal Ca^{2+} measurements and external Ca^{2+} fluxes. These fluxes were in order of greater magnitude than what is needed for supporting intracellular apical Ca^{2+} gradient. On the basis of these findings it was suggested that cell wall could be acting as a buffer for Ca^{2+}. The Pectin-methyl-esterases which are required for cross-linking unesterified pectins have been proposed to be controlling the availability of binding sites for Ca^{2+} on the cell wall. The above hypothesis is challenged by apical $[Ca^{2+}]_c$ measurements with aequorin (Messerli and Robinson 1997) which reveals that peak values are higher in about one order of magnitude than previous estimates that were taken by using Ca^{2+}-sensitive dyes. An alternative explanation to these different studies is that $[Ca^{2+}]_c$ influx regulation takes place by capacitative entry. Trewavas and Malhó (1997) suggested that the activity of ion channels in tip-growing cells might be regulated by levels of intracellular Ca^{2+} stores in a way that when stores are at full capacity the plasma membrane channels would close and vice versa.

9.3.2 cNMPs in Pollen

The cloning of a putative adenyl cyclase in pollen tubes has revealed motifs which are common to its fungal counterpart (Moutinho et al. 2001) as well as to proteins involved in disease responses. There is a role of cAMP in such responses (Cooke et al. 1994) and parallels have been drawn between growth of pollen tube in the style and the infection by fungal hypha. Although the cDNA was not of full length, it had caused cAMP accumulations upon expression in *E. coli,* and it was shown complementing a catabolic carbohydrate fermentation defect in an *E. coli cya*A mutant (Moutinho et al. 2001). The use of antisense oligos against the above cDNA or the antagonist treatment caused pollen tube growth disruption which suggests that adenyl cyclase is continually required for cAMP synthesis. This was also supported by the imaging study employing an adenyl cyclase activator, forskolin, which resulted in transient increase in cAMP, while an adenyl cyclase activator dideoxyadenosine resulted in temporary decline (Moutinho et al. 2001). These transitional changes in cAMP suggest the presence in pollen tubes of the degrading enzymes such as phosphodiesterases. Cyclic nucleotides were also found to play a role in controlling ion homeostasis in plants, for example, for the regulation of Ca^{2+} levels the cAMP appears to be involved (Volotovski et al. 1998). The $[Ca^{2+}]_c$ transients were experienced by the pollen tubes upon different treatments which putatively affect the cAMP levels. The caged release of ~1–2 μM cAMP declined the growth rates temporarily and also resulted in reorientation of growth axis of the pollen tubes. At the same time the elevation in $[Ca^{2+}]_c$ takes place in the apex and not in subapical region. In contrast, the application of cAMP externally resulted in the growth arrest and elevation of $[Ca^{2+}]_c$ in the both apical and subapical regions. These effects suggest the toxicity and ion flux perturbations in the pollen tubes due to elevation of cAMP, and this can be explained by the experimental evidence revealing the small release of cAMP upon flash photolysis. These findings suggest that cAMP pathways role in reorientation of pollen tubes acting together with Ca^{2+} control. However, in an experiment with membrane permeable cGMP, when the microneedle was placed near the growing tip of pollen tube, it failed to cause and show any response (Moutinho et al. 2001). Prado et al. (2004) has suggested the role of cGMP in the signaling cascade that is affecting growth regulation. However, the use of drugs in this study was shown disrupting the growth regulation, and this pleiotropic response hindered the isolation and specific testing of cGMP. Further tests and studies are thus required for evaluating the role of other CNs in pollen tube growth and reorientation.

9.3.3 CNGCs in Pollen

A crucial aspect of the study of CNs signal transduction is the identification and characterization of CNs receptors, such as Cyclic Nucleotide-Gated Channels (CNGCs) (Zagotta and Siegelbaum 1996) and cyclic nucleotide-dependent protein kinases (Shuster et al. 1985) that are thought to mediate several effects of CNs. It is well established that plant cells possess CNGCs transporting several ions such as Na^+, K^+, or Ca^{2+} (Gaynard et al. 1996; Li et al. 1994; Kurosaki 1997). The patch clamp experiments on mesophyll cells have revealed that by elevating cAMP (but not cGMP) in a stepwise manner, there was an increase in outward K^+ current only in the presence of a phosphodiesterase inhibitor (Li et al. 1994). Furthermore, in similar type of study the outward K^+ current was inhibited upon using protein kinase A peptide inhibitor. The presence of an inward K^+ channel that is modulated by cAMP was also shown (Kurosaki 1997), and in *Arabidopsis,* it was shown that the two specific KAT1 and AKT1 (inwardly rectifying K^+ channels) channel activities were modulated by cGMP (Gaynard et al. 1996). The Cyclic Nucleotide-Gated Channels respond to both cAMP and cGMP and are regulated by Ca^{2+} and were detected in barley aleurone plasma membranes (Schuurink et al. 1998), *Arabidopsis* (Leng et al. 1999), and tobacco plasma membranes (Arazi et al. 2000). It is a family with 20

related genes in *Arabidopsis*. CNGCs contain six putative membrane-spanning domains, a potential pore forming region between S5 and S6 and a CN-binding domain (CNBD) that partly overlaps with a CaM-binding site (CaMBS) in the cytosolic *C*-terminal region (Leng et al. 1999; Köhler et al. 1999; Arazi et al. 2000; Köhler and Neuhaus 2000). Although plant CNGCs show similarities in amino acid sequence and overall structure to the family of six membrane-spanning domains K^+-selective shaker family channels and to animal nonselective cation CNGCs, they differ in several key aspects. First, the pore sequence of plant CNGCs does not contain the GYGD K^+-selectivity filter sequence nor does it contain the sequence of the animal CNGC selectivity filter. Thus, the plant CNGC selectivity filter has unique properties in sequence and in function (Hua et al. 2003a). Therefore, plant CNGCs may differ from their animal counterparts in selectivity towards K^+, Na^+, Ca^{2+}, and possibly towards other cations (Kaplan et al. 2007). That CaM binds to CNGCs is quite clear since several of the channels were isolated as a result of screening for CaM-binding proteins. Additionally, the use of a putative CaM-binding domain was employed for identification with two hybrid methods (with CaM as bait) (Schuurink et al. 1998; Arazi et al. 2000). Direct binding of CaM to the channel was measured and the dissociation constant reported as 8 nM (Arazi et al. 2000). Thus CNGCs can potentially integrate signals from two intracellular transduction pathways, allowing cross talk between Ca^{2+} and CN signaling pathways. Elimination of the CaM and CN-binding region of the tobacco NtCNGC increased tolerance to lead and attenuated uptake of this metal (Arazi et al. 1999). A similar effect was observed with AtCNGC1 *Arabidopsis* mutant (Sunkar et al. 2000). Overexpression of the CNGC rendered the plants hypersensitive to lead with reduced sensitivity to nickel, suggesting that the channel acts as a port of entry for some metals. A direct demonstration that CNs enabled transport of Ca^{2+} was obtained when the *Arabidopsis* AtCNGC2 was expressed in oocytes (Leng et al. 2002) and showed that it would also complement a K^+ channel-deficient yeast (Leng et al. 1999); both CNs induced Ca^{2+} elevation inside cells. The AtCNGC2 is an inwardly rectifying channel that transports a number of cations and is unlike the highly specific K^+ (e.g., *KAT1*) channels previously described (Leng et al. 2002); as well as transporting Ca^{2+}, AtCNGC2 is blocked by external Ca^{2+}.

Based on microarray profiling, it was indicated that six CNGCs are expressed in mature pollen and ten are expressed during pollen development (Honys and Twell 2003); however, out of 20 CNGCs in *Arabidopsis thaliana,* five have been reported expressing in pollen (Takeuchi and Higashiyama 2011). Among these Cyclic Nucleotide-Gated Channels, CNGC18 has been reported to be involved in pollen tube growth (Frietsch et al. 2007; Chang et al. 2007) by possibly relaying CN signal into flux of Ca^{2+} and thus generating a tip-focused gradient of intracellular Ca^{2+} (Frietsch et al. 2007). The use of reporter gene construct β-glucuronidase showed tissue-specific expression of AtCNGC18, and the histochemical expression revealed its specific expression in pollen grains. This study clearly indicated the role of AtCNGC18 in pollen tube growth as the knockout mutants of CNGC18 exhibited disrupted pollen tube growth (Frietsch et al. 2007). However, recently we have identified two more CNGCs involved in fertilization, CNGC7 and CNGC8. Using reverse genetics, *cngc7* was shown to play a role in fertilization, revealed by the short siliques/reduced seed set phenotype found in two independent mutant alleles. However, the roles these proteins plays are not well understood. CNGC7 and CNGC8 show strong homology, suggesting functional redundancy. Functional redundancy was suspected due to pollen lethality of double homozygous plants. To uncover the potential role of CNGCs, the double homozygous mutant lines were complimented with either CNGC7 or CNGC8 GFP reporter constructs. The complemented lines were analyzed regarding morphology and pollen vitality. The GFP reporter constructs were used to assess the proteins subcellular localization, pollen tube growth in vivo, guidance, and reorientation capacities. Along with slightly shorter siliques,

seed set analysis showed a reduction in seed number at the base of the pistil, suggesting a reduction in pollen tube growth potential or a deficiency in tracking long distance tropism signals. We also obtained data suggesting that the Ca^{2+} levels in the cngc7/cngc8 pollen tubes (expressing a cameleon Ca-reporter constructs) were lower than in the wild type. We further collected evidence for a partial impairment of the reorientation capability of the CNGC7 and CNGC8 mutant cells when growing under the influence of an external electrical field. Taken together, our results identify CNGC7 and CNGC8 as the second example of a CNGC function that is required for successful fertilization in Arabidopsis and provide the first genetic evidence for partial functional redundancy among CNGCs in fertilization (Rehman et al. 2010). The partial redundancy of CNGC7 and 8 and providing essential function in initiating the pollen tube tip growth has been recently confirmed (Tunc-Ozdemir et al. 2013).

9.4 Future Prospects

The CNGCs are responsible for cytosolic Ca^{2+} conduction in plants during different signaling responses such as the defense and fertilization. Given the intricacy of signaling networks and the cross talk between multiple pathways, there are many questions which arise when we talk about a particular pathway in relation to a particular response. In the case of pathogen defense, it is yet to be elucidated that how the pathogenic perception is transduced into generation of cyclic nucleotides and which genes are responsible for the cyclic nucleotide synthesis during pathogenic processes. Further, it is not yet clear about the downstream events taking place in plants with the CNGCs-mediated Ca^{2+} rise in cells. In animals the cytosolic Ca^{2+} conductance takes place through the CNGC channels which exist in heterotetrameric form. These channels have either two or more than two CNGC genes encode for their subunits. However, this phenomenon is not yet clear in case of plants and how the polypeptides in plant CNGCs assemble to form the functional Ca^{2+} conducting inward channels. It has been suggested about the possibility of CNGCs from plants acting like the CNGCs from animals, and it has been argued that closely related CNGCs in a particular pathway may be part of the same CNGC or work together to form a same functional CNGC channel. The examples in this regard have been taken from the role of CNGC2 and CNGC4 which are similar, and the mutant studies have shown similar phenotypes in terms of HR impairment. Similarly in our case the two closely related CNGC 7 and CNGC8 mutants have shown similar phenotypes of pollen defects which impair fertilization. The redundancy shown in both cases may suggest them working as a single unit for functional Ca^{2+}-conducting channels. But there is much work to be done to ascertain these complicated issues related to signaling involving CNGCs in plants.

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Signaling in Response to Cold Stress

10

Tanveer Bilal Pirzadah, Bisma Malik,
Reiaz Ul Rehman, Khalid Rehman Hakeem,
and M. Irfan Qureshi

Abstract

World population is growing at a fast pace and is projected to reach 6.5 billion by 2050. At the same time, numbers of changes that are occurring in regular environmental parameters are posing threats to the agricultural productivity. Thus, feeding 6.5 mouths would indeed be a huge challenge. Besides the ever-growing human population and alterations in environmental scenarios, reduction in the area of land used for agriculture, declination of crop productivity, overexploitation of bioresources, mal-agricultural practices, and deleterious abiotic environmental stresses are leading to ecological imbalance. To reduce these losses scientists all over the world focus on novel strategies to enhance crop production in order to meet the increasing food demand and establish a balance among different ecological factors. The various abiotic stress conditions such as cold, temperature, drought and salinity cause noxious effects on plant growth and development ultimately affecting the crop productivity. Among various abiotic stresses, cold stress is one of the main environmental stresses that limits the crop productivity and geographical distribution of most valuable crop plants. However, plants show remarkable developmental plasticity to survive in a continually changing environment. Being sessile, plants have generated in the course of their development proficient strategies of tremendous response to elude, tolerate, or adapt to various types of environmental stress conditions including cold. The acclimatization to various abiotic stress factors is largely dependent upon

T.B. Pirzadah • B. Malik • R. Ul Rehman (✉)
Department of Bioresources, University of Kashmir,
Srinagar 190006, Jammu and Kashmir, India
e-mail: pztanveer@gmail.com; bisma767@gmail.com; reiazrehman@yahoo.co.in

K.R. Hakeem
Faculty of Forestry, Universiti Putra Malaysia,
Serdang 43400, Selangor, Malaysia
e-mail: Kur.hakeem@gmail.com

M.I. Qureshi (✉)
Proteomics & Bioinformatics Lab, Molecular Plant Lab, Department of Biotechnology, Faculty of Natural Sciences, Jamia Millia Islamia,
New Delhi 110 025, India
e-mail: mirfanq@gmail.com

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the activation of cascades of molecular channels involved in stress perception, signal transduction, and the expression of specific stress-related genes and metabolites. Understanding the pathway mechanisms by which plants recognize these stress signals and then transduce them to cellular machinery in order to stimulate adaptive responses is of crucial importance to crop biology. Here we summarize cold stress tolerance mechanism pathways in plants. The main significant points discussed in this chapter include (a) adverse effects of cold stress on plant physiochemical parameters, (b) sensing of cold temperature and involvement of various signal transduction pathways, (c) function of various compatible solutes or osmoprotectants, and (d) types and functions of different cold-responsive genes and transcription factors (TFs) involved in various cold stress tolerance mechanisms.

Keywords
Cold stress • Cold sensors • Signal transduction • Transcription factors • Metabolites

Abbreviations

ABA	Abscisic acid
ABREs	ABA-responsive elements
ADC	Arginine decarboxylase
AFPs	Antifreeze proteins
BADH	Betaine aldehyde dehydrogenase
bHLC	Basic helix-loop-helix
BL	Brassinolide
BRs	Brassinosteroids
bZIP	Basic leucine zipper
CaM	Calmodulin
CaMK	CaM-dependent protein kinases
CAMTA	Calmodulin-binding transcription activator
Cas	Cold acclimation-specific genes
CBF	C-repeat-binding factors
CBLs	Calcineurin B-like proteins
CDPKs	Calcium-dependent protein kinases
CIPKs	CBL-interacting protein kinases
CMO	Choline monooxygenase
CO	Choline oxidase
COR	Cold responsive
CRT	C-repeats
CS	Castasterone
DACC	Depolarization-activated Ca^{2+} channels
DAG	Diacylglycerol
DHN	Dehydrin
DNA	Deoxyribonucleic acid
DRE	Dehydration-responsive elements
DREB	Dehydration-responsive element binding
EBR	24-Epibrassinolide
ERD	Early responsive to dehydration
EREBP	Ethylene-responsive element-binding proteins
GA	Gibberellin
GB	Glycine betaine
GSA	Glutamate semialdehyde
HACC	Hyperpolarization-activated Ca^{2+} channels
HSPs	Heat shock proteins
ICE1	Inducer of CBF expression
KIN	Cold induced
LEA	Late embryogenesis abundant
LOV1	LONG VEGETATIVE PHASE 1
LTI	Low-temperature induced
LTST	Low-temperature-induced signal transduction
MAPKs	Mitogen-activated protein kinases
NAD	Nicotinamide adenine dinucleotide
OAT	Orn-d-aminotransferase
OsCDPK13	Oryza sativa CDPK13
OsSPDS2	Spermidine synthase gene
P5CA	δ-1-Pyrroline-5-carboxylate synthetase
P5CDH	P5C dehydrogenase
P5CR	P5C reductase

PA	Polyamines
PIP2	Phosphatidylinositol 4, 5-bisphosphate
PLC	Phospholipase-C
PLD	Phospholipase-D
PR	Pathogenesis related
ProDH	Proline dehydrogenase
RAB	Responsive to abscisic acid
RACE	Randomly amplified cDNA ends
RNA	Ribonucleic acid
ROIs	Reactive oxygen intermediates
ROS	Reactive oxygen species
SAM	S-adenosyl-1-methionine
SMDS	Spermidine synthase
SPMS	Spermine synthase
TFs	Transcription factors
THPs	Thermal hysteresis proteins
TPP	Trehalose-6-phosphate phosphatase
TPS	Trehalose phosphate synthase
W7	N-(6-Amonihexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride

10.1 Introduction

Nature nurtures all living organisms by providing suitable resources like air, water, temperature, light, and soil. Organisms exhibit efficient growth under optimal conditions of resources. However, imbalance in these nutritional and environmental factors forces the organisms to diversify their habitats. As a mere fact, plants are sessile organisms and hence cannot manage to escape from such adverse circumstances to move and sustain under suitable environment. The sessile nature of plants has led to developing an ability in plants to adapt to extrinsic, often calamitous environmental conditions. Plants might have abilities to combat various abiotic stresses like cold, heat, drought, salinity, light, etc., by various mechanisms. Such mechanisms are associated with signal perception to gene response. The more is the degree of adaptation and tolerance to stress in a plant, the higher is the yield of the crop. Such varieties which are attributed with a higher degree of tolerance and better yield are preferred in modern agriculture practices to encounter the growing demands for human food. Any environmental variations affecting crop productivity directly pertain to food security and the well-being of the human population. Abiotic stresses pose deleterious effects on crop productivity all over the world contributing to huge food and economic losses. At recent, these stresses are being aggravated due to the effect of global warming, imbalanced environment, and intensive agricultural practices. Among the various abiotic stresses, temperature is one of the most important abiotic stresses that restrict the plant growth and development, crop production, geographical distribution, and maturing season of many plant species. According to numerous reports, significant economic losses are caused annually by unfavorable temperature extremes and the increased frequency, degree, and severity of heat waves. The optimal growth temperature varies among species. Temperature stresses experienced by plants are generally categorized into three basic types (Sham and Aly 2012). The first category consists of frost tender plants, which have very little or no freezing resistance. Such plants are very sensitive to chilling conditions and thus cannot be cold acclimated in due course of growth and development. These chilling-sensitive plants show metabolic dysfunctions at the exposure of temperatures slightly below the optimum. The second category includes plants that bear some tolerance to frost resistance. These plants can tolerate various amounts of exposure to subfreezing temperatures. This category is characterized by a broad range of freezing tolerance ranging from slightly below freezing in broad-leafed summer annuals to −30 °C in perennial grasses. Freezing tolerance in plants is determined by various factors contributing collectively like stage of cold acclimation, rate and degree of temperature decline, and the genotype. The third category includes cold hardy plants generally temperate woody species. These plants can tolerate harsh environmental conditions after experiencing cold acclimation. Cold acclimation is a very complicated process by which the plants become capable to regulate and accommodate their metabolism to cold and enhance freezing tolerance in response to low nonfreezing temperature and thus able to pursue growth and development too. Acclimatization process involves alterations in various

biochemical and metabolic features like enhanced production and accumulation of compatible solutes or osmolytes such as soluble sugars, proteins, and low-molecular-weight nitrogenous compounds. Besides, there is also an alteration in fatty acid profile of membrane lipids (Walker et al. 2010). Another feature of this process involves a concerted regulation and expression of a number of cold-inducible genes (Trischuk et al. 2006). Identification of the fundamental genes that play a pivotal role in cold stress has thus become an area of interest because such genes find great application in genetic engineering to develop plants that possess better tolerance to cold temperature. Indeed, many of these genes responsible for cold tolerance have been identified, cloned, and characterized in many plant species (Thomashow 2001; Hakeem et al. 2012). Besides, a number of cold-induced (KIN) proteins also play a prominent role in acquiring freezing tolerance in plants (Seki et al. 2002). At a molecular level, plants respond to these abiotic stress conditions by reprogramming gene expression which in turn results in the adjusted metabolic alterations. Further, the effective modifications are dependent upon proper signaling during cold. Plants respond to these abiotic signals through a series of reactions which occur in cascades and are generally termed as signal transduction. These signal transduction pathways form a link that connects sensing mechanism by receptors and genetic response in terms of transcripts, proteins, and metabolites. Plants encounter cold stress by activating signal transduction cascades that ultimately check and regulate the physiological and biochemical responses essential for acclimatization (Zhu 2002; Hakeem et al. 2012). The initial step in switching on of these molecular responses is the recognition of the stress signal as it is perceived and then to communicate the message about it through signal transduction cascades. This signal is then transduced downstream and results in the production of second messengers including calcium, reactive oxygen species (ROS), and inositol phosphates which in turn modulate the intracellular calcium level. The signal is passed in cascades through various primary sensors which are localized in the membrane (Xiong et al. 2002) and are mainly proteins. These sensory proteins in turn communicate with their corresponding partners often initiating a phosphorylation cascade and target the main stress-responsive genes or the transcription factors (TFs) regulating these genes. The products of these stress genes then ultimately lead to plant adaptation and assist the plant to sustain and surpass the adverse conditions. Thus, the plant responds to these stresses as individual cells and synergistically as a whole organism. However, there is very little information known regarding these cold sensors in plants, and details of the early low-temperature signaling pathway are lacking (Beck et al. 2007). Various potential sensors in plants include calcium sensors, G-protein-coupled receptors, ROS sensors, Ca-dependent protein kinases (CDPKs), and two-component histidine kinases (Klimecka and Muszyńska 2007; Shao et al. 2007). Understanding the different pathway mechanisms by which plants transfer the signals to cellular machinery in order to stimulate adaptive responses is of fundamental importance to biology and thus finds immense potential in genetic engineering to generate more stress-tolerant plants just to cater to the needs of growing population in terms of food, clothes, wood, etc. Besides, there is an immediate need to deeply understand the expression of these genes and their involvement in cold signaling which ultimately allow us to clarify the ways in which plants adjust to the abiotic stress.

10.2 Adverse Effects of Cold Stress

Cold stress is one of the primary abiotic stresses that have a potential adverse effect on crop productivity, quality, and postharvest life depending upon the degree of severity, growth stage, and exposure duration. Every individual plant has to pass different growth stages and every stage is completed optimally under a unique set of temperature for proper development. However, a set of temperature conditions which are favorable for one species may not be suitable for other species. Lowering of temperatures will thermodynamically

reduce the kinetics of metabolic reactions. Exposure to low temperatures will shift the thermodynamic equilibrium such that there will be an increased likelihood that nonpolar side chain of proteins will become exposed to the aqueous medium of the cell. This will directly affect the stability and the solubility of many globular proteins (Siddiqui and Cavicchioli 2006). Such changes lead to the perturbances in the stability of proteins or protein complexes and also in plant metabolic regulations found mainly in membranes of cell, chloroplast, and mitochondrion. Among different stages of plants, the seedling stage is more susceptible to cold stress especially chilling. Plants show different symptoms in response to chilling stress such as desiccation, osmotic imbalance, discoloration, tissue breakdown, accelerated senescence, ethylene production, shortening of life span, and undergoing decay at a faster rate due to leakage of plant metabolites (Sharma et al. 2005) due to loss of membrane permeability. Chilling has also been known to cause disruption of deoxyribonucleic acid (DNA) strands, lowered enzymatic activity and specificity, rigidification of membranes, destabilization of protein complexes, stabilization of ribonucleic acid (RNA) secondary structure, production and accumulation of reactive oxygen intermediates (ROIs), destruction of photosynthetic pigments, and leakage across membranes (Nayyar et al. 2005). Chilling also affects mitochondria as it causes its swelling and degeneration (Gutierrez et al. 1992). It also causes matrix enlargement, shortening, and reduction in number of cristae that often leads to an oxidative phosphorylation reduction (Yin et al. 2009). Cold stress also involves some qualitative as well as quantitative fluctuation in membrane lipid composition (Matteucci et al. 2011). But the magnitude of fatty acid unsaturation and the content of phospholipids enhance during cold acclimation. Thus, membrane rigidness and thus stability and population of receptor proteins are considered to play a pivotal role in cold perception. Fluctuation in membrane rigidity is recognized by various membrane proteins which act as primary sensors or regulators (Los and Murata 2004). Reactive oxygen species (ROS) formation during temperature stress also causes disruption of electron flow in electron transfer chains and thus disturbs the redox potential of various metabolic pathways. Thus, ROS formation causes cellular injuries and apoptosis which eventually leads to death of plant due to impairment of photosystem II reaction center and membrane lipids (Suzuki and Mittler 2006). The lipids present in the membrane are generally composed of two types of fatty acids which include unsaturated (including mono- and polyunsaturated) as well as saturated fatty acids. Generally during chilling stress degeneration of membrane is caused by severe cellular dehydration, associated with ice crystal formation in intercellular locations. Thus, the accretion of ice formation in the intracellular spaces causes physical interruptions and wreckage of cells and tissues. Besides, the lipids containing saturated fatty acids possess a unique property, viz., they undergo solidification at a higher temperature as compared to unsaturated fatty acids. Thus, membrane fluidity is strongly influenced by relative content of unsaturated fatty acids (Steponkus et al. 1993). Therefore, temperature plays a vital role in changing the membrane dynamics from semifluid state to a semicrystalline state and is termed as the transition temperature. Chilling-sensitive plants possess higher transition temperature than chilling-resistant plants due to higher proportion of saturated fatty acid. However, the actual reason of freeze-induced injury to plants is the ice formation rather than low temperatures. This ice formation in plants initially begins in the apoplastic spaces because of relatively lower solute concentration (Fig. 10.1).

10.3 Response of Plants Towards Cold Stress

The response of plants to any abiotic stress signal is mediated by a cascade of reactions, collectively termed as signal transduction. Abiotic stresses trigger a multitude of physiological responses at molecular level. The responses are complicated and highly regulated, resulting in activation of signaling pathways and genes encoding proteins

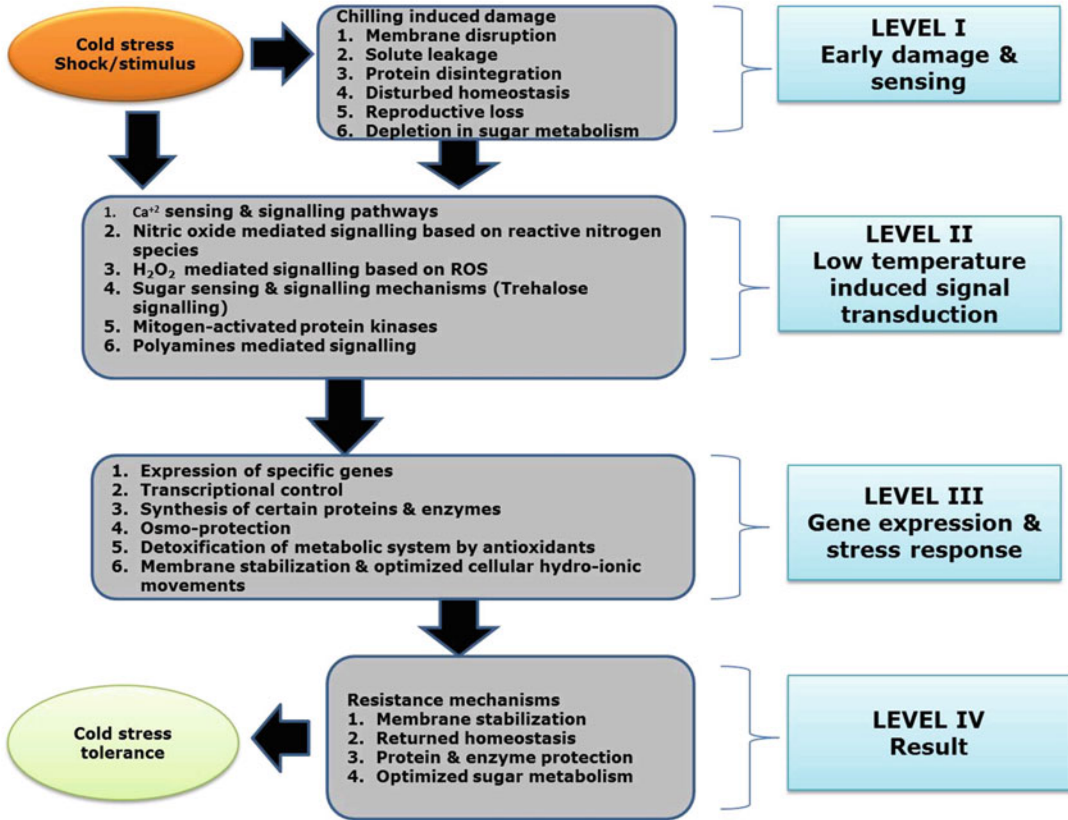

Fig. 10.1 A schematic presentation of four-tier mechanism of cold stress response and acquired tolerance in plants. Cold tolerance is also known as cold hardening or cold acclimatization and it is described as the development or increase in tolerance to cold temperatures over time by means of adaptive and resistive cellular mechanisms, which are stimulated in response to stressful cold temperature conditions

that act directly in stress tolerance. To combat such cold stress conditions, plants develop multiple mechanisms, including the accumulation of osmolyte molecules which include soluble sugars, proteins, low-molecular-weight nitrogenous compounds, proline, and glycine betaine (GB). Besides, there also occur changes in membrane lipids, primary and secondary metabolite composition, as well as changes in global gene and protein expression (Lissarre et al. 2010). Low-temperature-induced (LTI) signal transduction (LTST) has been elucidated in various steps: (a) sensing of low temperature, (b) transduction of signal into biochemical processes via secondary messengers such as Ca^{2+}, (c) activation/deactivation of kinases and phosphatases, (d) transduction of signals to the nucleus, (e) stimulation of particular genes in response to signal more accurately cold acclimation-specific genes *cas* genes, (f) expression of related transcripts, (g) synthesis of tolerant proteins and associated metabolites, and (h) improvement of freezing tolerance (Monroy et al. 1998).

10.4 Role of Cold Stress Proteins

Broadly, the types of proteins expressed in response to cold stress are antifreeze proteins (AFPs), dehydrins (DHN), late embryogenesis abundant (LEA) proteins, heat shock proteins (HSPs), chaperonins, pathogenesis-related (PR) proteins, and those related to transduction, transcription regulation, and signaling pathways.

10.4.1 Antifreeze Proteins (AFPs)

Antifreeze proteins (AFPs), another LT-responsive gene family, were first observed and characterized in polar fishes that dwell in waters where the temperature usually varies between −1 and −2 °C (DeVries 1971). Moreover, these proteins have also been discovered in many overwintering vascular plants (Venketesh and Dayananda 2008). Interestingly, these proteins get activated only after plants are exposed to low temperatures (Yaish et al. 2006). The cold stress proteins identified as β-1, 3-glucanase-like proteins, chitinase-like proteins, and thaumatin-like proteins and as polygalacturonase inhibitor proteins are synthesized in overwintering monocots in response to cold stress conditions and show better antifreeze activities (Seo et al. 2008). These proteins are released in the apoplast of the shoots including leaves and vegetative and reproductive whorls and are present in relatively large concentration (Antikainen et al. 1996) at these locations. Since formation of ice crystals poses serious threats associated with physical nonreversible damage, AFPs suppress the growth of an ice crystal by inhibiting the aggregation of water molecules to the growing faces of the crystal by a non-colligative mechanism. Due to the non-colligative nature of AFPs, these get adsorbed onto the surface of ice crystals and retard their growth proving beneficial in retention of cell membrane fluidity. Thus, instead of forming a single large ice crystal which causes fatal effects to the cells, more but smaller and slower growing crystals are formed. The antifreeze action of these proteins is generally regulated by Ca^{2+} ions, which are either discharged from pectin or bound to specific proteins (Janská et al. 2010). AFPs also possess a unique property of lowering the temperature that boosts freezing (ice crystal growth) than the temperature that promotes thawing that is why such hysteresis-related proteins are often considered as thermal hysteresis proteins (THPs). As extracellular freezing can cause cell dehydration, the down-regulation of aquaporins (water channels that regulate water efflux) may well be important for cold acclimation (Peng et al. 2008). Thus, antifreezing proteins play an important and prominent role in plants by directly interacting with ice and suppress freezing/mechanical injury by retardation of growth and recrystallization of ice (Griffith et al. 2005). Thus, AFPs are induced under the freezing temperatures and tend to interact with water to curtail the chances of ice crystal formation due to their special colligative properties.

10.4.2 Heat Shock Proteins (HSPs)

Heat shock proteins (HSPs) were earlier reported to be induced under high temperature and are playing thermo-protective roles. Though these proteins are generally concerned with high temperatures, numerous researchers proclaimed that these proteins also exhibit response towards other stresses too including low-temperature stress. In case of peach, synthesis of Hsp70-like proteins gets increased in response to cold stress (Renaut et al. 2008). Similarly, in another study, two members of the Hsp70 protein family that are equivalent to the *Arabidopsis* chloroplast Hsp70s were also found to be overexpressed at 5 °C. It has been revealed that these proteins play an essential role to protect the organisms from environmental stresses such as temperature stress and maintain their homeostasis (Morimoto et al. 1994). These proteins are not so prominent in the plants grown under favorable or optimum temperature but become prominent in concentration when plants are subjected to stress like heat shock (Lindquist 1986; Finka et al. 2011). Besides discovering the functions of HSPs, numerous studies show that the primary structure of HSPs is well conserved among all species ranging from bacteria and other prokaryotes to eukaryotes. The molecular size of HSPs varies, all of which are characterized by binding to structurally unstable proteins. These proteins are involved in various metabolic and physiological functions where they act as molecular chaperones (Boston et al. 1996). It is now well evident that HSPs/chaperones are responsible for thermotolerance, translation, proper folding, assemblage, and stabilization of proteins and membranes and can assist in protein refolding

under stress conditions (Sarkar et al. 2009). Further, these proteins also possess the ability to maintain and assist with the pace of various biochemical processes by means of maintenance or reestablishment of protein in native conformation and thus cellular homeostasis (Ahuja et al. 2010). In eukaryotes six different families of HSPs have been reported. These include HSP100, HSP90, HSP70, HSP60, sHSPs (small HSPs) and the Ubiquitin HSP8.5 group (Efeoglu 2009). HSPs like HSP 100, HSP 90, HSP 70, HSP 60, and the small HSPs (5 HSPs) are involved in protection of metabolic modules and newly made proteins from mis-folding and denaturation. A special form of HSPs, protease-type HSPs, degrades proteins that have been permanently damaged. Integral HSPs are able to protect and resume some aspects of cell components and metabolism of temperature damage (Yan et al. 2006).

10.4.3 Dehydrins (DHN)

At recent, it has been revealed that increase in level of hydrophilic proteins leads to the formation of amphipathic α-helix and is considered as an excellent protective response of plants towards cold stress (Eriksson et al. 2011). The dehydrin (DHN) involves a cluster of heat-stable, glycine or lysine-rich LEA proteins and gets stimulated when exposed to stress conditions such as cold stress that usually leads to dehydration. DHN has a role in stabilization of membranes and protection of other proteins from denaturation due to water loss induced by the stresses (Kosova et al. 2007; Bies-Etheve et al. 2008). Besides, DHN also acts as an antioxidant and molecular shield (Tunnacliffe and Wise 2007). Molecular mass of DHN varies over a wide range from 9 to 200 kDa and are thermostable in nature. Transgenic plants overexpressing DHN show better tolerance to low temperature than non-transgenic plants (Yin et al. 2006). In recent past, by using microarray technology the expression profile of some particular combination of genes encoding DHN proteins furnishes positive evidences regarding low-temperature and drought stress tolerance (Tommasini et al. 2008). More recently, proteomic investigations on soybean root revealed the production of DHN under drought stress (Alam et al. 2010). Zhang et al. (2010) reported that DHN also enhances resistance of peach fruits to cold stress. In barley, 13 DHN genes, dhn1 to dhn 13, have been reported to date. These proteins are often termed as cold responsive (COR), LTI, responsive to abscisic acid (RAB), KIN, or early responsive to dehydration (ERD). Accumulation of one of the cold-regulated chloroplast LEA III protein, viz., wheat WCS19, in transgenic *Arabidopsis* increased resistance to freezing stress, thus suggesting that LEA III proteins enhanced freezing tolerance (NDong et al. 2002). In one study a gene isolated from wheat (WCor410) encoding a LEA II protein was introduced in strawberry; the resulting transgenic plant shows enhanced tolerance against freezing and chilling stress after pretreatment with the acclimatization process (Hara et al. 2003) for low temperature. Structurally, DHN consists of three domains which are highly conserved. These domains are known as K, Y, and S segments (Allagulova et al. 2003). Besides, the number and order of the Y, S, and K segments define different DHN subclasses: YnSKn, YnKn, SKn, Kn, and KnS. Rorat (2006) reported that DHN-like SKn and K-type are directly involved in cold acclimation processes. Ubiquitous K-segment of DHNs shows similarity to a class A2 amphipathic α-helix, resembling the lipid-binding domain found in apolipoproteins (Campbell and Close 1997). Group 2 LEA proteins or DHN (Pfam 00257) are also found in algae and share a common K-segment present in one or several copies. Many DHN also contains an S-segment (polyserine stretch) that can undergo extensive phosphorylation and a Y-domain (DEYGNP), similar to the nucleotide-binding site of plant and bacterial chaperones. These DHN proteins not only exhibit a response to drought stress but also to other stresses such as cold, salinity, and hormonal stress especially ABA and methyl jasmonate. These DHNs also get accumulated in shoots and roots during the process of cold adaptation, especially in the plants tolerant to drought. However, some of their roles are known but several still remains to be worked out. However,

DHN does not catalyze any biochemical reaction in the metabolism. There are several reports showing that DHN proteins behave like antifreeze or cryoprotective molecules (Bravo et al. 2003; Puhakainen et al. 2004). Besides the abovementioned functions, they also play an essential role as osmoregulators and free radical scavengers in plants.

10.5 Role of Osmoprotectants

Accumulation of osmoprotectants is one of the very common responses of plants to a number of stresses which might otherwise imbalance osmoregulation. Similarly, during cold stress plants accumulate various low-molecular-weight compatible solutes that help the plant to survive by stabilization of proteins and membranes and contribute to maintain osmotic pressure of a cell. Such molecules are known as osmoprotectants or osmolytes or compatible solutes. These various molecules possess a short half-life period, i.e., they undergo a degeneration process once the stress conditions are surpassed. However, these osmolytes also serve as reservoir of nitrogen and energy. Generally throughout the evolution organisms living under extremely harsh environmental conditions have developed a protective mechanism by which they defend themselves against these environmental stresses, but many of the plants do not have the ability to combat such harsh conditions. Therefore, they undergo severe osmotic stress which results in detrimental modifications in cellular components. It is believed that osmoregulation would be the best approach for abiotic stress tolerance, especially if osmoregulatory genes could be triggered in response to drought, salinity, and high temperature (Bhatnagar-Mathur et al. 2008). Besides plants, other organisms also produce and accumulate these compatible solutes, viz., glycerol in yeast (Morales et al. 1990) and phytoplanktons (Ben-Amotz and Avron 1979), ectoine in *Halomonas,* trimethylamine N-oxide and urea in shallow-sea animals (Yancey et al. 2002). The polyols production such asglycerol, mannitol, sorbitol and sucrose in response to various abiotic stress have been reported in algae and certain halophytic plants (Yancey et al. 1982). Nelson et al. (1999) reported that stress-tolerant common ice plant, viz., *Mesembryanthemum crystallinum*, synthesizes and accumulates some specialized polyols like myoinositol, D-ononitol, and D-pinitol in the cytosol in order to combat harsh environmental conditions. These osmoprotectants include soluble sugars (trehalose, raffinose, stachyose, and saccharose), sugar alcohols (sorbitol, ribitol, and inositol), and low-molecular-weight nitrogenous compounds (i.e., proline and glycine betaine) (Walker et al. 2010). These, in association with DHN proteins, cold-regulated proteins (CORs), and HSPs, act to stabilize both membrane phospholipids and proteins, and cytoplasmic proteins; maintain hydrophobic interactions and ion homeostasis; and scavenge ROS; other solutes released from the symplast serve to protect the plasma membrane from ice adhesion and subsequent cell disruption (Chen and Murata 2008) of plant cells.

10.5.1 Carbohydrates

Sugars play a central regulatory role in many vital processes of photosynthetic plants besides serving the energetic function and are considered as important signals which regulate plant metabolism and development. It has been revealed that carbohydrate metabolism exhibits better and rapid low-temperature sensitivity than any photosynthetic pigments (Fernandez et al. 2012). Sugars play multiplex role in cold stress signaling as they are the efficient compatible solutes that help the plant to conserve the water inside cells, thus decreasing water accessibility for nucleation in the apoplast (Ruelland et al. 2009; Fernandez et al. 2010). In several studies it has been described that during cold acclimation concentration of soluble sugars (trehalose) increases dramatically including *Arabidopsis* (Miranda et al. 2007). Transgenic *Arabidopsis* with modulated sucrose level shows a direct relation between cold-induced modulation of sucrose metabolism and cold tolerance (Strand et al. 2003). The levels of sugar also increase during

the process of cold acclimation under artificial conditions (Hekneby et al. 2006). Studies conducted with mechanical approaches reveal that these carbohydrates stabilize the membranes by intercommunicating with polar groups of phospholipids and establishing hydrophobic bond interaction with proteins thus shielding the native structure and functioning state of membranes including chloroplasts and mitochondria, which are the major suppliers of active forms of oxygen. Besides, carbohydrates also act as scavengers of ROS. There is a close link between sugar signaling and hormone signaling, which regulates the growth, development, and stress response in plants (Zeng et al. 2011). Various sugars involved in maintenance and membrane integrity under cold stress include trehalose, fructans, raffinose, and sucrose.

10.5.1.1 Trehalose

Trehalose belongs to nonreducing disaccharides consisting of two molecules of glucose linked by an α, α (1–1) linkage that functions as a compatible solute. It is an important osmoprotectant/carbohydrate reserve found in various organisms like yeast, bacteria, fungi, insect, and vertebrates (Fernandez et al. 2010). Recently, it has been reported in a few desiccation-tolerant plants, indicating its function in acclimatizing to water stress because of its unique capacity to function as water substitute on the surface of macromolecules. Flowers (2004) reported that it might play an essential function in ROS scavenging and signaling cascade. Biosynthesis of trehalose is a two-step process and involves precursors like glucose-6-phosphate and uridine diphosphoglucose. The initial step involves the synthesis of trehalose-6-phosphate catalyzed by the enzyme trehalose-6-phosphate synthase (TPS). The resulting end product in turn is converted into trehalose by trehalose-6-phosphate phosphatase (TPP). In plants, trehalose-6-phosphate concentration is extremely controlled either by enzymes that directly metabolize it or by trehalase, which causes disruption of trehalose sugar. Trehalose sugar has also a special property to stabilize cell membrane whose fluidity diminishes during temperature downshift. Both trehalose-6-phosphate phosphatase activity and trehalose concentration were transiently increased after chilling (12 °C) stress, confirming that brief stimulation of trehalose biosynthesis is involved in early chilling stress response in rice (Pramanik and Imai 2005). Recently, Zhu et al. (2007) reported that overexpression of TPP, an enzyme in rice plant, conferred abiotic stress tolerance. Overexpression of TPS, another enzyme for trehalose biosynthesis, in rice also enhances environmental stress tolerance (Redillas et al. 2012).

10.5.1.2 Other Oligosaccharides

Besides trehalose, other oligosaccharides such as raffinose, stachyose, verbascose, fructans, and sucrose also provide greater application in providing resistance to plants against abiotic stresses including cold stress. These saccharides also show accumulation under stress conditions (Usadel et al. 2008). Fructans are the energy reserve resources in plants that can easily be circulated during periods of limited energy supply. The fructans find great application in cold stress signaling pathways because of some special unique properties like higher water solubility and tolerance to crystallization at freezing temperature (Livingston et al. 2009). Fructans are synthesized from its precursor, viz., sucrose, and the enzyme involved in this process is fructosyl transferase. The gene encoding the enzyme namely, fructosyl transferase is responsible for biosynthesis of fructans when introduced into tobacco and rice plants; the resulting transgenic plants show increased production of fructans and thus enhance its cold tolerance capacity (Kawakami et al. 2008). Besides acting as osmoprotectants, fructans also play a pivotal role to stabilize membranes by binding to the phosphate and choline groups of membrane lipids. This stabilization mechanism prevents membrane desiccation to enormous degree and water loss from desiccated membranes (Valluru and Van den Ende 2008). Moreover, fructans also stimulate other molecules resulting in the formation of alternate cryoprotectants (Valluru et al. 2008). These oligosaccharides have great significance in providing membrane protection and also possess free radical scavenging activity (Nishizawa et al.

2008). The gene OsUGE-1 is isolated from rice which encodes a UDP-glucose 4-epimerase when introduced in *Arabidopsis*; the transgenic plants show an increased level of raffinose and thus provide much tolerance against freezing temperature than wild species (Liu et al. 2007). During the process of cold acclimation, the genes that encode proteins responsible for synthesis of different types of sugars like sucrose and raffinose which are involved in various metabolisms such as sugar transport (β-amylase and SUC synthase) are accelerated in response to cold stress (Rekarte-Cowie et al. 2008). Furthermore, these sugar signaling molecules also control the expression of some COR genes like β-amylase (Kaplan and Guy 2004).

10.6 Low-Molecular-Weight Nitrogenous Compounds

Cold stress also results into an accumulation of low-molecular-weight nitrogenous compounds including proline, glycine betaine, and ectoine in plants. However, these compounds are widely induced in response to a number of stresses and cold stress is not very uncommon in this aspect.

10.6.1 Proline

Proline is a proteinogenic α-imino acid which plays an essential role in primary metabolism. It plays some significant roles in plants such as ROS scavenger and serves as a molecular chaperone to stabilize protein conformation (Szabados and Savoure 2010) besides acting as an important osmolyte in plants to mediate osmotic adjustment. Further, proline might act as an inducer of osmotic stress-related genes (Theocharis et al. 2011). Recently, Bashir et al. (2012) have shown that higher accumulation of proline might be an adaptation against stress to waive off the involvement of S-containing amino acids into osmoregulation rather involving them in synthesis of stress proteins. Proline is also a potential inhibitor of apoptosis, i.e., programmed cell death. Other functions of proline include its ability to regulate cytosol acidity, maintain NAD^+/NADH ratio, and enhancement of photochemical activity of PSII (Kishor et al. 2005). Proline synthesis might be triggered by de novo synthesis or its decreased degradation or both. However, there are some opinions that it might be the degradation product of proline-rich proteins which, however, needs experimental support. Some work shows that biosynthetic pathway of proline is regulated by light and osmotic stress. However, its catabolism is stimulated by dark as well as stress conditions (Szabados and Savoure 2009). Biosynthesis of proline takes place in the cytosol or chloroplast. The precursor used for the synthesis of proline is glutamate and ornithine. Recently, it has been reported that there is an enhanced production of proline synthesis and decreased degradation when plants are subjected to stress conditions like cold stress (Jonytiene et al. 2012). Glutamate is changed to glutamate semialdehyde (GSA) by an enzyme δ-1-pyrroline-5-carboxylate synthetase (P5CA). GSA in turn is converted to pyrroline-5-carboxylate (P5C) which is further reduced by enzyme P5C-reductase (P5CR) to proline. However, degradation of proline takes place in mitochondria with the help of two enzymes, viz., proline dehydrogenase (ProDH) and P5C-dehydrogenase (P5CDH) and converted to glutamate. The alternative precursor for proline biosynthesis includes ornithine which is transaminated by P5C by Orn-d-aminotransferase (OAT), an enzyme present in mitochondria (Verbruggen and Hermans 2008). Nanjo et al. (1999) produced transgenic *Arabidopsis* by incorporating ProDH gene which encodes the enzyme protein dehydrogenase using antisense technology. The resulting transgenic plant shows more accumulation of proline and better tolerance to freezing temperature. The production of proline increases in low-temperature-insensitive plants like *A. thaliana* when exposed to cold stress. This shows a positive correlation between endogenous protein production and cold stress tolerance (Kaur et al. 2011). Interestingly, like in tobacco plants an *Arabidopsis* does not accumulate proline concentration during heat stress; however, induced proline concentration makes plants more sensitive to heat (Lv et al. 2011).

Recently, transgenic tobacco has been produced in which overexpression of GK74 and GPR results in the increased production of proline (Stein et al. 2011). Thus, proline seems to play roles under cold stress varying from osmoregulation to radical quenching. It would further be interesting if proline is found to mediate signaling or serve as a signaling molecule during cold stress.

10.6.2 Glycine Betaine

Glycine betaine (GB), another compatible solute or osmoprotectant, is a quaternary ammonium compound in which nitrogen is fully methylated. It has been discovered in a number of life forms, viz., bacteria, hemophilic archaebacteria, marine invertebrates, and mammals. However, in case of plants it is not universal (Chen and Murata 2011). The most interesting feature of GB is that it is an amphoteric compound and is electrically neutral over a wide range of pH. It possesses higher water solubility and capable to interact with hydrophilic and hydrophobic regions of macromolecules (Sakamoto and Murata 2002). GB helps the plants by behaving like a shield and efficiently protects PSII, stabilizes thylakoidal and other membranes, and mitigates oxidative damage (Chen and Murata 2011). High concentration of GB is found in the chloroplast where it protects the thylakoid membrane and maintains photosynthetic efficiency. However, at lower concentration it maintains quaternary structure of enzymes and complex proteins (Peleg et al. 2011) helping in running of metabolism during cold. One of the other important roles of GB is to stabilize transcriptional and translational machineries. On biosynthetic aspects, choline and glycine are the main precursors of GB and its synthesis takes place in chloroplasts. Biosynthesis of GB involves two steps and is catalyzed by different enzymes, viz., choline monooxygenase (CMO) which acts on choline and converts it into betaine aldehyde and another enzyme betaine aldehyde dehydrogenase (BADH) which converts betaine aldehyde to betaine. There are two kinds of BADH which are found in mangrove halophytes *Avicennia marina* and possess high efficiency rate to convert betaine aldehyde to betaine (Hibino et al. 2001). Recently, with the help of genetic engineering, the genes encoding the enzymes responsible for GB synthesis when introduced into *Arabidopsis* and tobacco, the transgenic plants accumulated GB in their cells and built resistance against freezing temperatures. This shows a positive correlation between GB accumulation and abiotic stress tolerance (Bansal et al. 2011). In another study, it has been found that overexpression of the gene for *Arthrobacter* choline oxidase (CO) causes an accumulation of GB in plant cells of *Arabidopsis* and tobacco and thus provides higher resistance to stresses such as low and high temperature. Chen et al. (2011) also reported that when GB is exogenously supplied to plants, it enhances stress tolerance in addition to helping growth and survival.

10.6.3 Polyamines (PAs)

Polyamines (PAs) are low-molecular-weight aliphatic molecules and are positively charged at cellular pH. PA is composed of two or more primary amino groups. These PAs play important roles in various physiological processes like flower, meristem, and gametophyte development (Zhang et al. 2011b). Besides, they are also involved in alleviating oxidative stress and enhancing photochemical efficiency of PSII (Kim et al. 2002; Hussain et al. 2011). During stress conditions, PAs play a pivotal role as secondary messengers and regulate various biochemical and molecular processes (Alcázar et al. 2010). PAs are biosynthesized from its precursor putrescine. The putrescine is converted into spermidine and spermine. This reaction is catalyzed by two distinct enzymes, viz., spermidine synthase (SMDS) and spermine synthase (SPMS). Both enzymes utilize decarboxylated S-adenosyl-1-methionine (SAM) as an aminopropyl donor. Though very little information is known regarding the involvement of PA in cold stress (Gill and Tuteja 2010), it has been reported that stress results in the production and

accumulation of PA indicating its integral role in stress tolerance (Liu et al. 2011a). Cuevas et al. (2008) reported that plants combat any stress conditions involving PA; the putrescine is synthesized and modulates ABA biosynthesis at transcriptional level. Thus, these PAs function as signaling molecules for phytohormone biosynthesis. It has been reported that when rice plant is exposed to long-term chilling stress, spermidine synthase gene *OsSPDS2* gets upregulated (Imai et al. 2004). Similarly, in case of *Thlaspi arvense*, transcripts for SAM synthase get upregulated upon cold stress (Sharma et al. 2007). Recently, Alet et al. (2011) observed that overexpression of arginine decarboxylase (ADC) led to higher production of putrescine and enhanced its freezing tolerance. A cDNA clone of spermidine synthase was prepared from *Cucurbita ficifolia*, and the gene was then introduced into *Arabidopsis* using *CaMV35S* promoter. The resulting transgenic plant shows significant enhancement in spermidine synthase activity and spermidine content. Besides, the transgenic plant shows better tolerance to freezing temperatures and other abiotic stresses (Kasukabe et al. 2004). The levels of putrescine and spermidine were found higher when wheat was subjected to stresses like cold stress, osmotic stress, and ABA stress. In addition to that, ABA treatment also shows enhancement in levels of a polyamine, cadaverine (Kovacs et al. 2010).

10.7 Role of Different Cold Sensors in Signal Transduction

Like other organisms plants also possess sensor molecules which play indispensable role in receiving different signals including for cold. These signals are then passed and decoded into a proper cellular message. However, to date very little information is known regarding these sensors. These protein-based sensors are generally found in plasma membrane and detect conformational change in microdomain of membrane. The various potential cold sensors are described below:

10.7.1 Calcium Channels

Calcium plays an essential role in cold signal transduction pathways. It is one of the most ubiquitous cellular second messenger which acts as a mediator of stimulus–response coupling in the regulation of physiological process (Kudla et al. 2010). The calcium is stored in plastids usually in the form of calcium-oxalate crystals. The first response of plants towards cold stress involves actin cytoskeletal rearrangement. This in turn activates Ca^{2+} channels and thus increases Ca^{2+} levels. Three Ca^{2+}-gated ion channels exist in the plasma membrane of plants. These include MCC, the depolarization-activated Ca^{2+} channel (DACC), and the hyperpolarization-activated Ca^{2+} channels (HACC) (Sanders et al. 2002). Such channels possess unique features to detect different physiochemical stimuli such as cold, heat, and ABA (Carpaneto et al. 2007).

Ca^{2+} channels present in the plasma membrane and in intracellular membranes do not alone regulate the cytosolic Ca^{2+} concentrations. The function of Ca^{2+} sensors in transcription regulation is interconnected to their subcellular localization (nucleus or cytosol). For the CaMs, CMLs, and CDPKs, localization is probably widespread inside the cell (DeFalco et al. 2010). The cytosolic Ca^{2+} levels are brought back to the resting level by means of primary and secondary transporters present in membrane. Active transport of Ca^{2+} is brought about by Ca^{2+}/H^+ antiporters and Ca^{2+} ATPases, regulators of Ca^{2+} efflux from cytosol, which modulate Ca^{2+} concentrations in the cytosol, load Ca^{2+} into intracellular compartments, and supply Ca^{2+} to organelles to support metabolic reactions. Several genes encoding Ca^{2+} ATPases have been cloned from *Arabidopsis* (Sanders et al. 1999), but their function in cold stress is not clear yet. However, Ca^{2+} ATPase activity enhances in winter rye leaves in response to low-temperature stress (Puhakainen et al. 1999). A Ca^{2+}/H^+ antiporter gene has also been isolated and characterized from *Arabidopsis*. Transgenic tobacco plants overexpressing *Arabidopsis* Ca^{2+}/H^+ antiporter gene exhibit susceptibility to cold stress indicating that antiporter activity is pivotal for acclimatization to cold

stress (Hirschi 1999). A major function of Ca^{2+}/H^+ antiporters and Ca^{2+} ATPases may be to dismiss Ca^{2+} signals by restoring cytosolic Ca^{2+} levels to pre-stimulus values (Sanders et al. 1999). Both Ca^{2+} ATPase and Ca^{2+}/H^+ antiporter seem to be subject to regulation. However, the signals that induce and control these efflux systems are unknown. An elevation in Ca^{2+} level in response to low temperature results in rapid and temporary closure of plasmodesmata which regulates cell-to-cell transport of ions and small molecules (Holdaway-Clarke et al. 2000). Taken together, control of both influx and efflux systems of Ca^{2+} in cold signaling is of equal importance allowing sensitizing and desensitizing the cell for cold-induced Ca^{2+} signal, which acts as a second messenger.

10.7.2 Phospholipases

Phospholipases also play an essential role as sensor molecules in cold signaling pathways. To enhance higher level of freezing tolerance, both compositions of phospholipids and their interaction with other proteins are important (De Palma et al. 2008). The various phospholipases in plants include phospholipase-C (PLC), phospholipase-D (PLD), and phospholipase-A_2 (PLA2). One of the unique features of phospholipase A_2 is that it gets rapidly activated under cold stress, wound, and other stresses besides other phospholipases and mediates subsequent events for cold signaling and cell protection (Chapman 1998). The PLC is the common signaling pathway that accumulates cytosolic Ca^{2+} levels. The PLC gets activated by means of various cell surface receptors like G-protein-coupled receptors and receptor-tyrosine kinases. PLC causes hydrolysis of phosphatidylinositol 4, 5-bisphosphate (PIP_2) into IP_3 and diacylglycerol (DAG) (Fig. 10.2). IP_3 and DAG then act as second messenger. IP_3 releases Ca^{2+} from internal stores. It has been reported that IP_3 shows rapid response to hyperosmotic stress (Takahashi et al. 2001). PLD is involved to participate in various stress signaling pathways. This sensor acts as a structural and signaling link between plasma membrane and the cytoskeleton

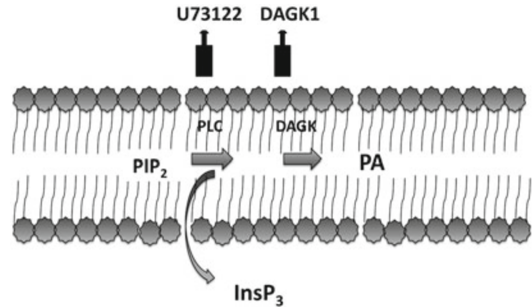

Fig. 10.2 Role of PLC in response to cold stress in cell suspension culture of *Arabidopsis*

in tobacco and *Arabidopsis* (Gardiner et al. 2003). PLD acts as an inhibitor of phosphatidic acid which in turn controls the activity of some particular cold-regulated (COR) genes (Vergnolle et al. 2005). PLD catalyzes phospholipids such as phosphatidylcholine, phosphatidylethanolamine, or phosphatidylglycerol into phosphatidic acid. It has been reported that in case of *Arabidopsis* cell suspension culture, the cold stress leads to the production of phosphatidylbutanol or phosphatidylethanol in the presence on n-butanol and ethanol, respectively (Ruelland et al. 2002). This shows that PLD gets activated under cold stress. Initially, the response to cold stress is very fast. Twelve PLD isoforms have been identified in *Arabidopsis*. Li et al. (2004) reported that overexpression of the gene that encodes PLD enhances the plant capacity towards low-temperature stress.

10.7.3 Histidine Kinases

The *Arabidopsis* genome possesses >600 RLK-encoding genes, some of which are implicated in the perception of environmental signals during the life cycle of the sessile plants. Histidine kinases are also membrane-localized kinases and perceive osmotic stress and plant hormones (Osakabe et al. 2013). The two-component histidine kinases play an active role as low-temperature sensors in plants. Several histidine kinases have been cloned and shown to be implicated in sensing hormonal and abiotic stresses such as cold stress. A hybrid histidine kinase

AtHK1 has been cloned from *Arabidopsis* (Urao et al. 1999). AtHK1 has been found to be accelerated in response to low temperature, high salinity, and dehydration. Structurally AtHK1 contains two hydrophobic transmembrane regions adjacent to a putative extracellular domain in the N-terminal half, suggesting functional resemblance with the yeast osmosensor SLN1. The function of the *AtHK1* protein is to recognize cold signal and then transduce it to the nucleus through a series of phosphorylation cascades.

10.7.4 Receptor Kinases

Receptor-like kinases (RLKs) are transmembrane proteins with an N-terminal extracellular domain and a C-terminal kinase domain. These kinases are involved in various physiological and developmental processes such as plant growth and metabolic reactions and also participate in various environmental stresses. RLKs recognize extracellular signals at the cell surface and activate the downstream signaling pathway by phosphorylating specific target proteins (Tanaka et al. 2012). In *Arabidopsis* about 600 receptor-like protein kinase (RLK) genes have been reported (Tichtinsky et al. 2003). Among these, greater than 400 are for transmembrane proteins with C-terminal cytoplasmic serine/threonine kinase domains and N-terminal extracellular domains. They act as a putative low-temperature sensor. The function of these membrane-spanning domains is to transmit extracellular signals into intracellular target molecules. A large portion is found in a putative leucine-rich repeat (LRR) subfamily of receptors. RPK1 is an *Arabidopsis* LRR-RLK that is activated not only by ABA but also by abiotic stresses, such as dehydration, high salinity, and cold (Hong et al. 1997).

10.8 Cold Signal Transducers in Plants

Calcium is considered as secondary messenger that participates to play a main role in cold signal transduction pathways (Klimecka and Muszyńska 2007). When plants receive cold signal, calcium gets accumulated in the cytosol that enables the plant to resist future cold stress in an efficient way. During cold stress Ca^{2+} concentration increases in cytosol by activation of ligand-activated Ca^{2+} channels. When cytosolic Ca^{2+} level increases, it either gets diffused from the cytosol or directly introduced into the nucleus in response to stimuli. Inside the nucleus, it directly interacts with TFs and regulates their activity (Kim et al. 2009). The message of Ca^{2+} signatures is then decoded into proper cellular response by means of specific Ca^{2+} decoders which regulates gene expression. These different Ca^{2+} signatures can be distinguished on the basis of different Ca^{2+}-binding proteins and protein kinases associated with it. In recent studies, it has been observed that in *Arabidopsis*, when exposed to cold stress, cytosolic Ca^{2+} level increases rapidly. This is mainly due to transient influx of Ca^{2+} from extracellular stores (Knight et al. 1996). Effective Ca^{2+} signatures are generated only in specific tissues or organs. For example, under cold stress Ca^{2+} influxes occur in the whole plant, while in case of drought stress, it is generated only in the roots (Knight and Knight 2001). Through different techniques, it has been observed that expression of few KIN genes, viz., CRT/DRE-controlled *COR6* and *KIN1* gene of *Arabidopsis*, is greatly influenced by cytosolic Ca^{2+} level (Chinnusamy et al. 2010). When Ca^{2+} influx is blocked by some Ca^{2+} chelators (BAPTA) and Ca^{2+} blockers (La^{3+}), expression of these cold-inducible genes (*Cas15*) gets inhibited and thus reduces their ability to cold stress. However, on treating cells with A23187, a Ca^{2+} ionosphere, it shows rapid influx of Ca^{2+} and thus enhances the *Cas15* gene expression. Therefore, such cells resist the temperature of about 25 °C (Monroy and Dhindsa 1995). A transgenic *Arabidopsis* and tobacco was developed expressing Ca^{2+}-sensitive luminescent protein aequorin and shows rapid increment in cytosolic Ca^{2+} level in response to cold stress. This suggests that there is a unique signature of the cytosolic Ca^{2+} for various stresses. Reddy and Reddy (2004) observed that there is a concrete association KIN Ca^{2+} influx and accumulation of cold-induced transcripts. Generally, there are four

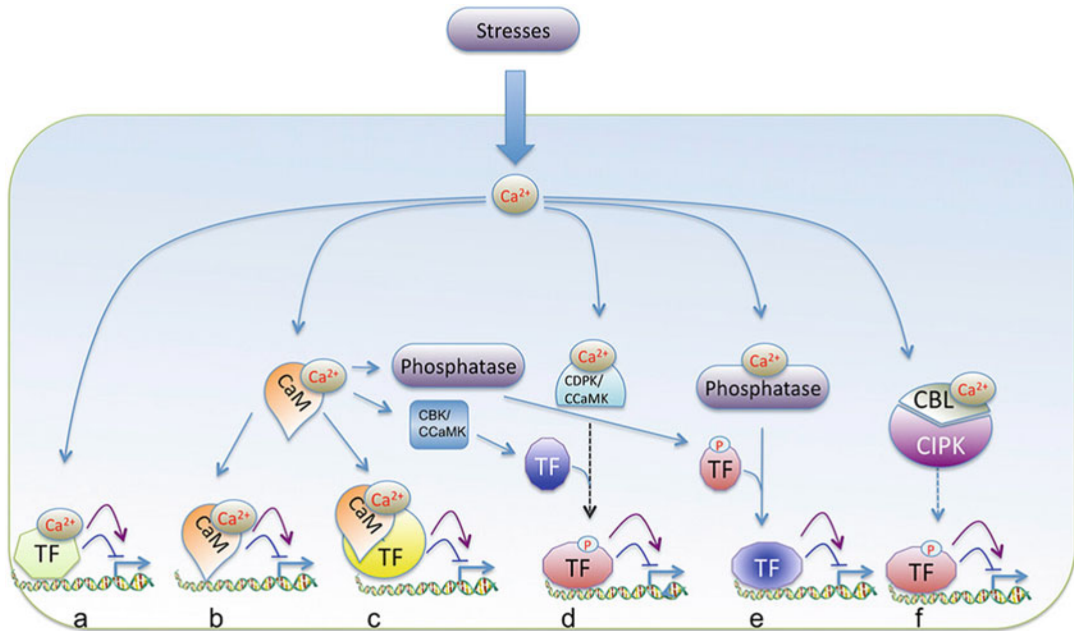

Fig. 10.3 Signal-induced transient influx of cellular calcium ([Ca^{2+}] cyt and/or [Ca^{2+}] nuc) can regulate transcription by different mechanisms. Elevated Ca^{2+} levels result in its binding to a Ca^{2+} sensor, which directly binds to specific DNA sequences and modulates gene expression (*a* and *b*). Activated calcium sensors (Ca^{2+}/CaM or Ca^{2+}/CML) interact with DNA-binding proteins and modulate their activity resulting in altered transcription (*c*). Finally, an elevated level of calcium activates a protein kinase (CDPK, CBK, and/or CCaMK) either directly or through CaM or a protein phosphatase, which in turn phosphorylates or dephosphorylates a TF, respectively, resulting in activation or repression of transcription (*d* to *f*). *Solid arrows* indicate pathways with experimental evidence. Pathways lacking evidence are represented by *broken arrows*. *Purple arrows* indicate activation of gene expression; *blue lines* with a *horizontal line* represent repression (Source: Reddy et al. 2011)

types of calcium sensor proteins present in plants. These include calmodulin (CaM), CaM domain-containing protein kinases, calcineurin B-like proteins (CBLs), and CBL-interacting protein kinases (CIPKs). However, these sensors can be categorized into two major classes. The first class includes "relay sensors" such as CaM and calcineurin B-like proteins without any responder domain. These relay sensors bind to Ca^{2+} and undergo conformational changes which in turn modulate the activity of various target proteins. The second class includes "responders" such as protein kinases and phospholipases which possess effector domains through which the Ca^{2+} signatures are passed on to downstream targets (Zeinolabedin and Seyyed 2012). This shows that rise in cytosolic Ca^{2+} alone or in combination with CaM could stimulate these responders. The link between calcium signaling and cold induction of the C-repeat-binding factor (CBF) pathway reveals that CaM-binding transcription activator (CAMTA) factor binds to a regulatory element of *CBF_2* promoter gene. As CAMTA being TFs, thus they act directly in the transduction of cytosolic Ca^{2+} signals induced by low temperature into downstream regulation of gene expression (Doherty et al. 2009). Recently, Yang et al. (2010) reported that a novel calcium/CaM gene *CRLK1*, which regulates receptor-like kinases, was found to play an essential role in cold stress (Fig. 10.3).

10.9 Components of Calcium Signaling

Calcium is an essential second messenger in plant signaling networks. Many environmental and developmental stimuli induce an increase in

cytosolic calcium to trigger different physiological responses. The specificity of Ca^{2+} signaling is achieved by a combination of distinct calcium signatures that are generated by specific calcium channels, pumps and transporters, and diverse calcium sensors that differ by their expression pattern, sub-cellular localization, substrate specificities and calcium sensitivities. These Ca^{2+} signatures are received, recognized, decoded, and transmitted to downstream responses by a complex cascade of Ca^{2+}-binding proteins that function as Ca^{2+} sensors. Plants possess an extensive and complex array of such Ca^{2+} sensors that convey the information presented in the Ca^{2+} signatures into phosphorylation events, changes in protein-protein interactions, or regulation of gene expression. Prominent Ca^{2+} sensors like calmodulins (CaM), calmodulin-like proteins (CMLs), calcium-dependent protein kinases (CDPKs), calcineurin B-like proteins (CBLs), and their interacting kinases (CIPKs) exist in complex gene families and form intricate signaling networks in plants that are capable of robust and flexible information processing (Hashimoto and Kudla 2011). The various but important components of calcium signaling would be discussed below.

10.9.1 Calcium-Dependent Protein Kinases (CDPKs)

CDPKs belong to the class of plant protein kinases which contain kinase domain and a Ca^{2+}-binding domain. These are serine/threonine-rich kinases with a C-terminal CaM-like domain with up to four EF-hand motifs that can directly bind Ca^{2+}. These protein kinases are specifically expressed in plants and considered as important sensors which show a better response against abiotic stress including cold (Klimecka and Muszyńska 2007). CDPKs play a duel role, viz., they act as Ca^{2+} sensors as well as possess kinase activity. The various subfamilies of CDPKs include CDPK, CCaMK (Ca^{2+} or Ca^{2+}/CaM-regulated kinases), CaMK (CaM-dependent protein kinases), CRK (CDPK-related kinases), and CIPKs. Many CDPKs are found to be accelerated by cold stress in different plants as these protein kinases serve as receptors for Ca^{2+} signal. In a study, when alfalfa cell suspension culture was provided with W7, an antagonistic of CDPKs and CaM, the culture loses its capacity to withstand cold stress (Monroy et al. 2007). Abbasi et al. (2004) described the role of *OsCDPK13* (*Oryza sativa* CDPK13) gene against cold stress. In rice genome, 31 CDPK genes have been identified. Ray et al. (2007) carried out an extensive research analysis of CDPKs in rice; it was found that OsCPK4, OsCPK5, and OsCPK13 (OsCPK7) get upregulated upon cold stress. Besides, Hrabak et al. (2003) reported that in case of *Arabidopsis*, there are 34 genes for CDPKs. This indicates that activity of CDPKs is regulated by multigene families. It reveals that the gene *OsCDPK13* gets accelerated in response to cold stress and gibberellin (GA) but suppressed by drought, salinity, and ABA. The transcription of the gene *OsCPK13* (*OsCDPK7*) is induced by chilling stress, but when the same condition are provided to another gene *OsCPK17*, its transcription gets downregulated (Wan et al. 2007). The protein which is encoded by the gene *OsCDPK13* is increased in 2-week-old leaf sheath and callus, and when it was subjected to cold stress it gets phosphorylated.

10.9.2 Mitogen-Activated Protein Kinases (MAPKs)

MAP-kinase cascades also participate in cold signal transduction and are encoded by a large family of serine/threonine protein kinases (Saidi et al. 2011). These MAP kinases are activated by phosphorylation of the conserved threonine and tyrosine residues that are located close to kinase domain VIII in all MAPKs under the action of MAPK kinases (MAPKK) which themselves are phosphorylated by MAPKK kinases (MAPKKK), raf and mos proteins (Fig. 10.4). A given dual-specificity MAPKK can only activate a specific MAPK and cannot functionally substitute other MAPKKs. A particular set of three functionally interconnected protein kinases (MAPKKK-MAPKKMAPK) forms the primary module of an MAPK pathway. ROS which are produced during metabolism is an integral part of cold signaling.

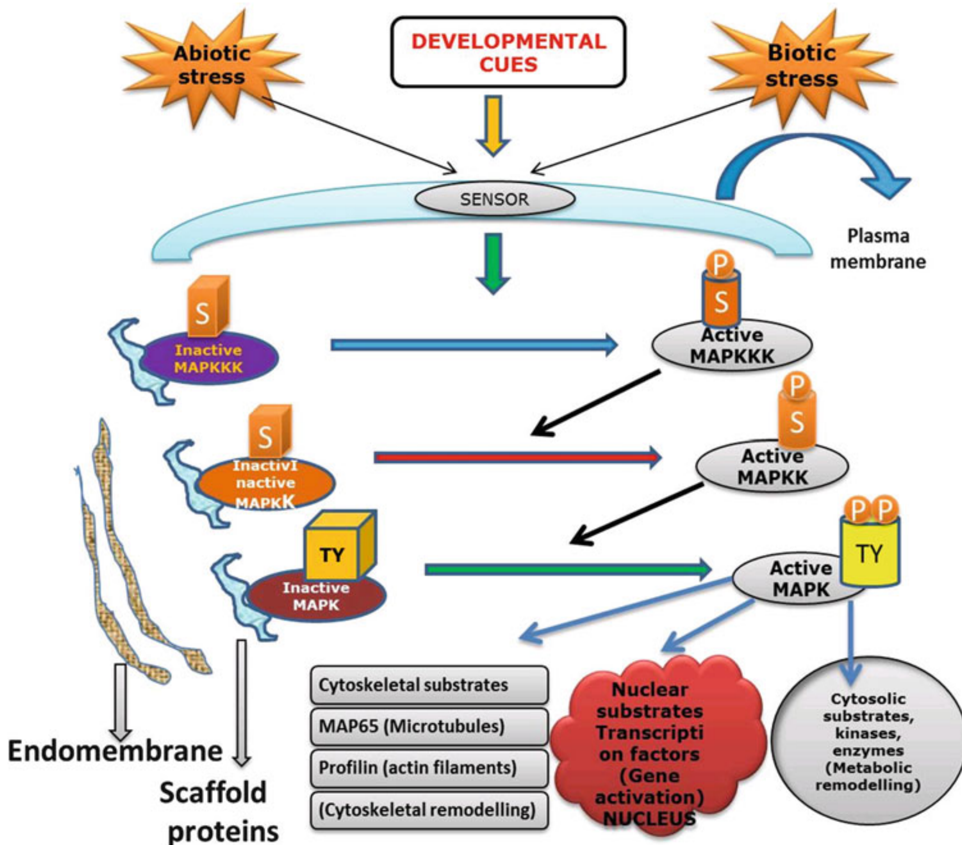

Fig. 10.4 Simplified scheme for MAP kinase signal transduction cascades. An extracellular signal is received by a membrane-located receptor. The activation of the MAP kinase module (MAPKKK activates MAPKK, activates MAPK) by the receptor may occur via several intermediate steps and by different routes. The active MAP kinase may activate other protein kinases and phosphorylate cytoskeletal components or translocate to the nucleus and activate TFs giving rise to expression of specific genes. *Arrows* indicate activation

These reactive oxygen species (ROS) indirectly activate the TFs by targeting the components of MAP-kinase cascades. ROS such as H_2O_2 may alter calcium expression (signatures) and stimulate mitogen protein kinases (MAPK) and redox-responsive TFs. It has been reported that ROS activates a MAP-kinase cascade in *Arabidopsis*. The cascade includes *ANP1* (MAPKKK) *AtMPK₃*, and *AtMPK₆* and its positive regulator, nucleoside diphosphate kinase 2 (Moon et al. 2003; Teige et al. 2004). Gene *NPK1* that is homologue to *ANP1* was isolated from tobacco plays an essential role in cold signaling (Kovtun et al. 1998). Besides, this gene is also involved in developmental processes. Moreover, Kovtun et al. (2000) produce transgenic plants overexpressing *ANP1* and exhibiting extra resistance to freezing, heat shock, and salt stress. The genome of the widely studied plant *Arabidopsis thaliana,* for example, encodes about 1,000 protein Ser/Thr kinases, including about 60 MAPKs and nearly 400 membrane-associated receptor kinases that phosphorylate Ser or Thr residues; a variety of protein phosphatases; scaffold proteins that bring other proteins together in signaling complexes; enzymes for the synthesis and degradation of cyclic nucleotides; and 100 or more ion channels, including about 20 gated by cyclic nucleotides. Knight and Knight (2001) described three kinds of MAPKKKs in *Arabidopsis thaliana*. These include (a) CTR1, (b) ANP1-3, and (c) *AtMEKK*. Among these, *AtMEKK* are expressed in response to various abiotic stresses including cold stress. Recent data reveals a

positive role for MAPK-based signaling in stress-induced proline accumulation (Zhang et al. 2011a). MAPK kinase ZmMKK4 promotes proline as well as soluble sugar accumulation under stress conditions (Kong et al. 2011). Various genes that encode MAP kinases show upregulation when subjected to cold stress such as *AtPK6*, *AtPK19*, *AtMPK3* (MAPK), and *AtMPKK1* (MAPKKK) (Mizoguchi et al. 2010; Yoshida et al. 2010). Another gene found in alfalfa is homologue to MMK4 and also shows upregulation when subjected to cold stress (Jonak et al. 1996). Recently, a MAPK that was stimulated by heat shock (HAMK) was identified and was found to be different from the cold-activated MAPK (SAMK). It has been reported that activation of HAMK and SAMK was triggered by apparent opposite changes in membrane fluidity coupled with cytoskeletal remodeling, Ca^{2+} influxes, and the action of Ca^{2+}-dependent protein kinases (CDPK). Recently, Wang et al. (2010) reported that in maize ABA-induced production of H_2O_2 activates two other MAP kinases, viz., ZmMPK3 and ZmMPK5. Among these MAP kinases, ZmMPK3 shows sensitivity to various signaling molecules like jasmonic acid and salicylic acid. Besides, it also gets activated by means of various abiotic stresses such as cold, drought, salinity, or UV light stresses. In another study, the MAP-kinase gene found in rice, viz., OsMEK1, encodes MAPKK, and its transcripts are induced in anthers by a 12 °C treatment for 48 h. *OsMEK1* induction is also observed in shoots and roots of seedlings treated at 12 °C for 24 h. No *OsMEK1* induction is observed in seedlings treated at 4 °C (Wen et al. 2002).

10.10 Abscisic Acid (ABA)

Plants growing in temperate and frigid areas are largely exposed to cold stress for comparatively longer periods. However, at such regions too plants have to complete their life cycles for their progeny continuation. In the plants growing at the said locations or during the times of facing the winters, chilling and freezing temperature produces numerous dysfunctions at the molecular level like membrane impairment, ROS formation, protein denaturation and production, and accumulation of toxic products. To counteract such deleterious effects, plants develop various mechanisms such as alteration of gene expression, production of osmoprotectants, generation of antioxidants, biosynthesis of phytohormones like abscisic acid and ethylene jasmonic acid (Kang and Saltveit 2001). ABA is considered as a crucial signaling molecule that provides resistance to plants against cold stress. The shielding mechanism of ABA against chilling stress is associated to its capability for stabilizing the water status by enhancing root hydraulic conductivity and stomatal closure, despite of the above facts; ABA also induces and accumulates antioxidant enzymes in order to slow down the ROS production which is generated during low-temperature stress. Besides, ABA also modulates polyamine levels (Kumar et al. 2008; Liu et al. 2011a, b). ABA is a small soluble molecule which is synthesized in almost all cells, but it transports from roots to shoots. Since production of ABA generally occurs during stress conditions, it is considered as a stress hormone (Mahajan and Tuteja 2005). Further, this molecule moves comparatively freely in plants which make it a desired candidate as a secondary signaling agent. Cold stress also enhances endogenous ABA concentration in plants but to a much lesser extent. Exogenous application of ABA to plants increases their freezing tolerance. This shows that many COR are also ABA responsive (Rabbani et al. 2003). More than a thousand types of genes responsible for cold stress have been unraveled through transcriptomic analyzing techniques that are differentially regulated by ABA, and these ABA-mediated changes in gene expression translate to dominant modifications in proteome expression.

10.11 Brassinosteroids (BRs)

Brassinosteroids (BRs) involve a group of polyhydroxy steroidal phytohormones (Hayat and Ahmad 2011). These hormones are structurally equivalent to animal and insect steroidal hormones. BRs play key roles in growth and development, germination, and senescence. Besides, BRs play a crucial role in developing resistance in plants

towards cold stress by promoting plant growth and maintaining chlorophyll content. Besides, the BR hormones also assist in developing tolerance to elevated temperatures by maintaining protein synthesis machinery (Krishna 2003). Among various natural BRs, brassinolide (BL) is the first known biologically active phytohormone (Fujioka and Yokota 2003). The first known BR, viz., BL, was isolated from the pollen of *Brassica napus,* and over forty other allied compounds have now been recognized in different plant extracts. Biosynthesis of BRs occurs through different metabolic pathways which include early or late C-6 oxidation routes. The precursor for the biosynthesis of BRs is campesterol which is initially transformed into campestanol. The campestanol is further converted into castasterone (CS) which in turn is converted into BL. Recently with the help of proteomic analysis, the signal transduction channels of BRs from receptor kinases to diverse TFs have been illustrated and described in detail (Kim et al. 2012). *CYP90A2* gene isolated from mung bean is involved in brassinolide biosynthesis. This gene is strongly suppressed by chilling stress and thus arrests the growth due to lowering of BRs. However, exogenous application of 24-epibrassinolide (EBR) causes plants to recover its growth. It has been reported that in *Arabidopsis* plantlets, when exposed to cold temperature (2 °C) prior treated with 24-EBR, the transcript accumulation of CBF1, LT78, and COR47 was found higher than plantlets not treated with EBR (Kagale et al. 2007) (Fig. 10.5).

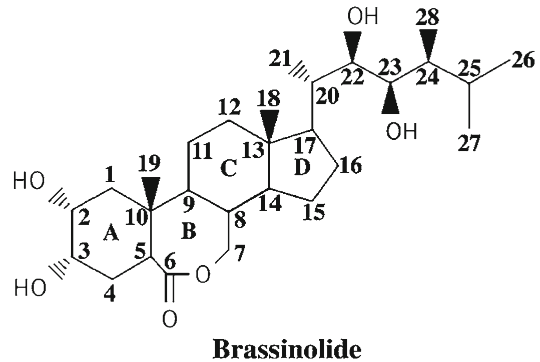

Fig. 10.5 The structure of brassinolide, a commonly occurring BR with high biological activity, showing *numbered positions*. In natural BRs, hydroxylation can occur in *ring A* at positions 3, and/or 2, and/or 1; also found are epoxidation at 2-, 3-, or a 3-oxo-group. In *ring B*, alternatives are 6-oxo- and 6-deoxo- forms. In the side chain methyl-, ethyl-, methylene-, ethylidene-, or *nor*-alkyl groups can occur at 24-, and the 25-methyl series is also represent

10.12 Cold Stress and Transcription Factors (TFs)

The process of acquiring tolerance to chilling temperatures can be achieved by exposure of plants to positive low temperature with gradual duration. This process is known as cold acclimation. However, it has also been reported that cold acclimation is also achieved when plants are subjected to stress conditions or treating by ABA. This is due to the fact that these stress conditions play a vital role to induce stress-related genes and the product which provide tolerance against these stress conditions. Various TFs that alleviate cold signaling have been reported in *Arabidopsis*, and several other homologues of these factors have also been recorded in other plant species (Solanke and Sharma 2008). Recently, it has been reported that in the plant cells these TFs are already present at normal growth temperature, but when plants are subjected to cold stress, these recognize the CBF promoters and induce their expression (Zarka et al. 2003; Akhtar et al. 2012). Promoter analysis of the cold-responsive genes (COR) revealed that they contain dehydration-responsive elements (DRE) or C-repeats (CRT) (Stockinger et al. 1997). Akhtar et al. (2013) cloned and characterized cold-inducible C-repeat-binding factor genes from a highly cold-adapted ecotype of *Lepidium latifolium*. Various COR genes (COR78/RD29A, COR47, COR15a, and COR6.6) have been reported from *Arabidopsis* that encode LEA-like stress proteins. These genes are influenced by cold signal, dehydration, or stress hormone ABA. The expression of COR genes is regulated by both ABA-independent and dependent pathways (Chinnusamy et al. 2003) (Fig. 10.6).

10.12.1 CBF Pathway

Numerous TFs known as C-repeat-binding factors (CBF) or DREBs that facilitate cold signaling have been reported in *Arabidopsis*. These TFs act

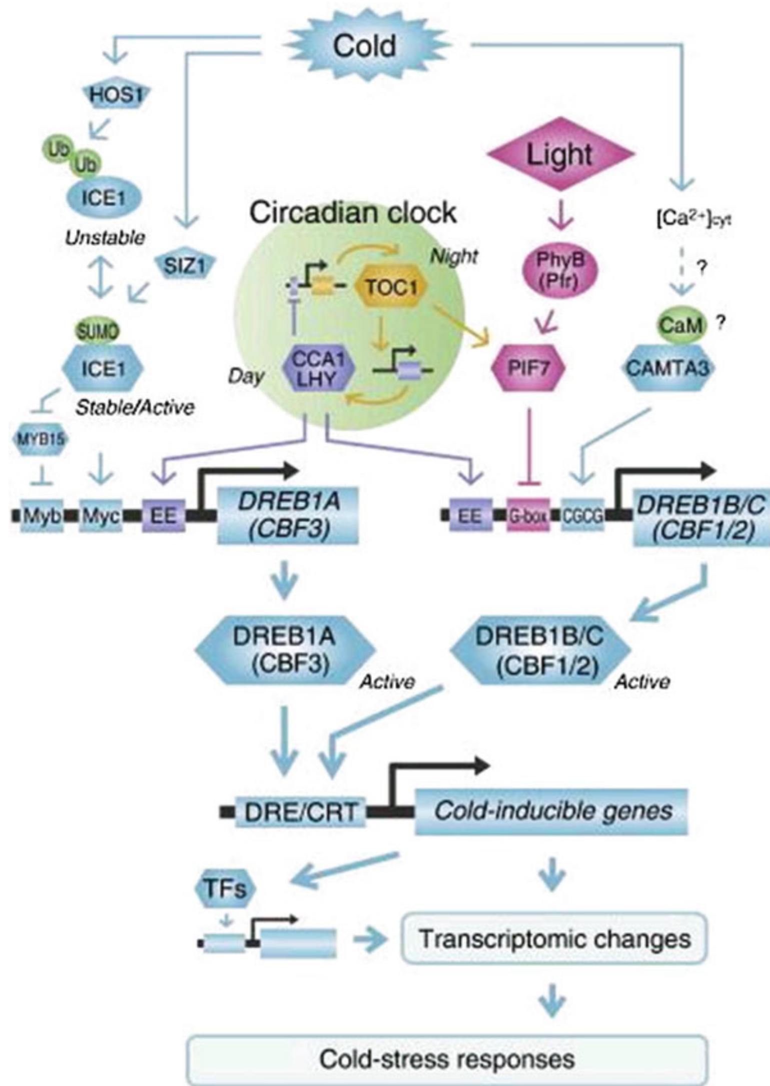

Fig. 10.6 Transcriptional regulation of cold-inducible DREB1s/CBFs in *Arabidopsis*. The activity of DREB1s/CBFs is mainly regulated at the transcriptional level. The transcription of *DREB1s/CBFs* is under the control of low-temperature signals and circadian/light signals. Expressed DREB1/CBF proteins bind to DRE/CRT and activate transcription of target cold-inducible genes, including TF, and thus bring about transcriptomic changes, which eventually cause cold stress responses. *Ub* ubiquitin, *CaM* calmodulin, *Myb* Myb-recognition sequence, *MYC* MYC-recognition sequence, *EE* evening element, *CGCG* CGCG-Box, *TFs* TF (Mizoi et al. 2012)

as molecular switches to induce the expression of specific genes responsible for cold, drought, and other stresses, respectively. So, it is clear that DRE-TFs are the main locus where different stress-induced pathways converge. Thus, it is concluded that DRE acts as a master TF and has the ability to integrate the information gathered from various stress stimuli. Moreover, due to this capability it plays a vital role in cross talk of stress signaling pathways. These TFs belong to the ethylene-responsive element-binding proteins/APETAL A2 (EREBP/AP2) family and DREB subfamily (Chen et al. 2009). Subfamily DREB is categorized into six subgroups, viz., A-1 to A-6. Subgroup A-1 includes DREB1-/CBF-like genes that are influenced by lower temperature and stimulate the expression of various cold stress-responsive (COR) genes. Subgroup A-2 includes

DREB2-like genes and plays an essential role in osmotic stress-responsive gene expression (Nakashima et al. 2009). Gilmour et al. (2004) recorded three cold-inducible genes such as CBF1/DREB1b, CBF2/DREB1c, and CBF3/DREB1a in *Arabidopsis*. However, these C-repeat (CRT)/dehydration-responsive element-binding factor genes (CBF1-3) are transcription activators involved in governing the plants response to low temperature (Fernando et al. 2012). The cold induction of CBF1–3 is repressed by STRS proteins (RNA helicases) and ZAT12, a transcriptional repressor. ZAT12 is itself induced by cold, may be through an ICE-like protein. Besides, these CRT/DRE elements comprise core conserved sequence (CCGAC), which is responsible to stimulate transcription under cold stress signals. A-5 subgroup members such as PpDBF1, GmDREB2, and GhDBF1 find great application in genetic engineering to enhance the stress tolerance of crops. In several studies, it has been recorded that ectopic overexpression of some CBFs resulted in both activation of target genes and improvement in freezing or dehydration tolerance of transgenic plants (Haake et al. 2002). Recently, one new member of A-5 subgroup of DREB family was discovered and isolated from soya bean with the help of RACE (Randomly amplified cDNA ends) technique. This includes a DREB orthologue, GmDREB3. By means of Northern blotting technique, the expression of GmDREB3 in soya bean seedlings was stimulated following cold stress exposure treatment for 0.5 h and was not observed after 3 h. The analysis reports of GmDREB3 revealed that it could be involved in the early cold response as compared to DREB2. In *Arabidopsis RD29A* gene was isolated which has the capacity to provide tolerance against cold and drought stress. In case of tomato overexpression of CBF1 increases its chilling tolerance by enhancing *CATALASE 1-gene-expressing* enzyme activity and oxidative stress tolerance (Hsieh et al. 2002). Badawi et al. (2008) identified several CBF genes that are classified into 10 subgroups. Overexpression of At CBF1/3 enhanced tolerance against cold, drought, and salt stress in *Brassica* species, wheat, and rice (Oh et al. 2005).

Another TF termed as inducer of CBF expression (*ICE1*) that acts as a key receptor of cold-induced gene expression is present upstream of CBF. ICE1 is an MYC-type basic helix-loop-helix (bHLC) TF that has the capability to bind with MYC-cis element in the DREB1a/CBF3 promoter region and enhances the expression of DREB1a/CBF3 which ultimately stimulates the expression of various downstream genes, leading to a significantly increased resistance to chilling and freezing temperatures (Chen et al. 2009). Constitutive expression of CBFs and *COR* genes significantly increases resistance in plants towards cold stress. Recently, it has been revealed that in case of heterologous *Arabidopsis* system the overexpression of some wheat *ICE1* genes is also involved in providing resistance against freezing temperature. Ice1 mutant plants are afflicted during cold acclimation and are defective in cold-regulating expression of CBF3 and its target *COR* genes (Chinnusamy et al. 2003). Besides, DREB1c/CBF2 negatively governs the expression of DREB1b/CBF1 and DREB1a/CBF3 (Novillo et al. 2004). Therefore, *Arabidopsis* mutant cbf2 (suppressed CBF2/DREB1c-gene) possesses superior quality to tolerate freezing temperature than wild-type ones before and after cold acclimation and is found to be more resistant to other stresses such as dehydration, cold, and salt stress. Another R2R3-type MYB-TF, AtMYB15, was also found to communicate with ICE1. It is reported that ICE1 negatively regulates MYB15 as indicated from the increased MYB15 transcript level in ice1 mutants compared to wild-type plants under cold stress. Recently, with the help of forward genetic analysis in *Arabidopsis*, two more TFs like osmotically responsive genes 9 (*HOS9*) and *HOS10* have been observed and are essential for basal freezing temperature. The *HOS9* and *HOS10* genes encode homeodomain (present in nucleus) and an R2R3-type MYB (*AtMYB8*) TF, respectively. However, their transcript levels are not cold responsive. Loss-of-function mutations in these genes cause substantial decrement in basal and acquired freezing temperature. Interestingly, the mutants

show potent or earlier cold induction of several CBF-target genes, like *RD29A* and *COR15A*, but no effects are seen on the expression of CBFs. It seems that *HOS10* could regulate ABA-mediated cold acclimation (Chinnusamy et al. 2007). LONG VEGETATIVE PHASE 1 (*LOV1*) encodes a NAC-domain TF. At 4 °C, the expression of COR25 is enhanced in *LOV1-overexpressing* plants but reduced in loss-of-function lov1 mutant. The expression at 4 °C of the *CBF* genes does not seem affected either by *LOV1* mutation or overexpression. Loss-of-function lov1–4 plants are more susceptible to freezing temperature than wild plants, while plants overexpressing *LOV1* have a higher constitutive freezing tolerance. Interestingly, *LOV1* negatively regulates the expression of *CONSTANS* (CO). *co-2* mutants are also freezing tolerant. This is consistent with the phenotype associated with LOV1 overexpression and suggests that CO, a well-known floral promoter, may also be involved in freezing tolerance. However, whether the effects of lov1 mutation on *COR15A* expression and freezing tolerance occur through CO is not known (Yoo et al. 2007). While *LOV1* positively regulates *COR15A* expression, other TFs have been identified that negatively regulate the expression of *CBF* target genes. A comprehensive account about structure, function, and roles of CBF has recently been provided by Akhtar et al. (2012).

10.12.2 ABA-Dependent Cold Signal Pathway

When plants are subjected to stress-like conditions such as low temperature, there is transient accumulation of ABA concentration because of the induction of freezing temperature and onset of cold acclimation. ABA finds great application in providing cold stress tolerance. However, many researchers reported that cold stress does not induce the synthesis of ABA but is primarily produced and accumulated in response to other stresses such as drought and high-salinity stress (Shinozaki et al. 2003). It has been reported that when plants are exogenously treated with abscisic acid at normal growth temperature, it enhances the plant's resistance towards chilling and freezing stress. Besides, it also causes novel protein synthesis and induction of a subset of cold-responsive genes. Recently, through microarray analysis it has been reported that CBFs govern only about 12 % of the cold-responsive transcriptome. Rest part of the cold-responsive genes (*COR*) is controlled by non-CBF TF (Fowler and Thomashow 2002). In the promoter region of *COR* genes, there are ABA-responsive elements (ABREs). The major cis-acting element are ABA-responsive elements with a consensus sequence C/TACGTGGC that confers ABA responsiveness to many genes when more than one copy is present. Two ABRE motifs are important in the ABA-responsive expression of the *Arabidopsis* gene *RD29B* (Sakuma et al. 2002). Uno et al. (2000) reported that the class of bZIP TF, ABRE-binding proteins (AREBs or ABFs), can bind to ABRE and induce ABA-dependent gene expression. ABF-encoding genes (ABF1-4) are themselves regulated by ABA and exhibit differential regulation by various abiotic stresses; ABF1 gets stimulated by cold, whereas ABF2 and ABF3 get stimulated by high salt concentration and ABF4 by cold, high-salinity, and drought stress conditions. In case of ABA-deficient mutant strains, viz., los5/aba3 of *Arabidopsis*, the genetic analysis reports reveal that ABA plays an essential role in regulating osmotic stress gene expression. Besides, such mutant strains (also named aba3/freezing sensitive 1 (frs1)) also show higher susceptibility to freezing stress. Chinnusamy et al. (2007) reported that one of the mutant los5 plants shows a notable decrement in the expression of cold and osmotic stress induction of genes.

10.13 Biotechnological Approach to Enhance Cold Resistance in Plants

Biotechnology plays an essential role in developing novel pathways that can be used to develop stress-tolerant plants. With the help of

genetic engineering, a large number of gene encoding proteins have been isolated and characterized that are involved in many stress signaling pathways. The current focus of research throughout the world involves novel genetic engineering techniques to develop a cold-resistant variety of plants. When plants are subjected to cold stress, a sequential cascade of genes are induced, the products of which may either directly protect against stress or further control the expression of other target genes. Genetic engineering has unlocked many mysterious ways to enhance cold stress in plants by the introduction or elimination of a gene or genes that act as checkpoints to regulate a specific trait. These plants have one or more novel genes from stranger or their wild relatives, which overexpress and regulate the functioning of metabolic process in a positive manner against stressful temperatures. It also provides unique opportunities for renovation in genetic potential of plants in the form of development of distinct crop varieties that are more resistant to abiotic stresses with increased nutritional level. The analysis of transgenic plants overexpressing one or other genes provides us an understanding of basic mechanism of functioning of stress genes during cold stress exposure (Tayal et al. 2005). Table 10.1 shows the list of transgenic plants produced for cold stress. The current challenges in developing cold-resistant plants include:

- To identify and characterize the cold sensor checkpoints and innovative signaling pathways responsible for abiotic stresses
- Understanding the basic cellular mechanism of intercellular as well as intracellular interaction among stresses
- Identification of the basic fundamental factors that play an essential role between abiotic stress responses and various developmental processes
- To address how different environmental stress signals are processed and communicated to other parts of the plant body

10.14 Conclusion and Future Perspective

The study of plant temperature interactions is of great relevance with respect to the current global climatic changes. The current review covers the different mechanisms and crucial players of signal transduction which the plant develops tolerance against cold stress. Cold stress is a critical stress factor limiting the productivity and distribution of many plant and crop species. Low temperature is initially recognized by plasma membrane either due to modifications in membrane fluidity or with the help of sensory proteins like Ca^{2+}-permeable channels, histidine kinases, receptor kinases, and phospholipases that finally lead to cytosolic Ca^{2+} influx. These signals are then transduced to the nucleus in order to switch on transcriptional cascades. Thus, understanding the mechanism of different signaling networks; role of TF, genes, and their products in regulating cold stress response; and cross talk among various signaling components should remain an area of intense research activity in the near future. As every abiotic stress involves multigenic traits therefore, a multi-interdisciplinary approach involving physiological and biochemical analyses aided by proteomic- and genomic-based platforms should be followed in order to develop novel methods of analysis just to acquire better knowledge of gene expression. The information gathered through these studies finds great application in genetic engineering to develop transgenic plants that possess better tolerance to various abiotic stress conditions without showing any growth and yield penalty. Future research for acquiring the cold stress tolerance would greatly be based on utilization of high-throughput techniques developed in recent times, in combination with conventional genetic and breeding protocols and techniques. The clues that we have in hand need to be further evaluated to extract exhaustive information regarding the structure, function, and mobilization of cold stress-tolerant genes with an eco-friendly manner to feed billions of mouths.

Table 10.1 A list of transgenic plants produced for cold tolerance showing the mobilized gene and effects imparted by them

Plant	Transformed gene	Effect of gene engineering	References
Oryza sativa	*TERF2*	Increased accumulation of osmotic substances and chlorophyll, reduced ROS and MDA content and decreased electrolyte leakage	Tian et al. (2011)
O. sativa	*OsRAN2*	Maintained cell division, decreased proportion of cells with intra-nuclear tubulin and formation of a normal nuclear envelope under the cold condition	Chen et al. (2011)
A. thaliana	*MbDREB1*	Drought, salt, cold tolerance	Yang et al. (2011)
L. perenne	*DREB1A/CBF3*	Drought and freezing tolerance	Li et al. (2011)
O. sativa	*MYBS3,* Single DNA-binding repeat MYB-TF	Repressed the well-known DREB1/CBF-dependent cold signaling pathway in rice, and the repression appears to act at the transcriptional level	Su et al. (2010)
Zea mays	*ZmMPK3*	Cold, drought, UV rays, salinity, heavy metal and mechanical wounding	Wang et al. (2010)
M. Truncatula, R. chinensis	*MtDREB1C*	Freezing tolerance	Chen et al. (2010)
A. thaliana	*RAP2.6*	Hypersensitive to ABA, salt, osmotic and cold stress (*Arabidopsis*)	Zhu et al. (2010)
N. tabaccum	Wheat *TaSOD1.1* and *TaSOD 1.2* genes	Increased SOD activities and decreased MDA content, lessened degree of over-oxidation of the cellular membrane system the enhancement of physiological functions	Hai-Na et al. (2009)
N. tabaccum	*OsSPX1*	Better seedling survival and reduced cellular electrolyte leakage, decreased total leaf Pi content and accumulation of free proline and sucrose, accumulation of free proline and sucrose	Zhao et al. (2009a, b)
A. thaliana	*DgDREB1A*	Drought and freezing tolerance	Tong et al. (2009)
T. aestivum	DRE-binding TF gene, *GhDREB* from *Gossypium hirsutum*	Improved tolerance to drought, high salt, and freezing stresses through accumulating higher levels of soluble sugar and chlorophyll in leaves after stress treatments	Gao et al. (2009)
O. sativa	*OsMYB3R-2* TF	Higher transcript levels of several G2/M phase specific genes, including *OsCycB1;1, OsCycB2;1, OsCycB2;2,* and *OsCDC20.1,* increased cell mitotic index, level of cellular free proline was increased	Ma et al. (2009)
O. sativa	*OsTPP1*	Trehalose synthesis	Ge et al. (2008)
O. sativa, A. thaliana	*OsDREB1F*	Drought, salt, and cold tolerance	Wang et al. (2008)
N. tabacum	*OsDREB1B*	Oxidative, drought, freezing tolerance	Gutha and Reddy (2008)
O. sativa	*OsMYB3R-2*	Stress tolerance	Dai et al. (2007)
H. vulgare	HvCBF4	Increased drought, salt and freezing tolerance (rice)	Oh et al. (2007)
N. tabacum	*FAD7*	Omega-3-fatty acid desaturase gene; survival in chilling conditions	Khodakovskaya et al. (2006)
B. napus	BNCBF17	Freezing tolerance	Savitch et al. (2005)

(continued)

Table 10.1 (continued)

Plant	Transformed gene	Effect of gene engineering	References
N. tabacum	AtDREB1A	Freezing and drought tolerance	Kasuga et al. (2004)
C. intybus	*Nicotiana tabaccum* Osmotin (*TbOsm*)	Accumulation of osmoprotectant proline in response to salt stress	Rehman (2003)
			Abdin et al. (2002, 2004)
A. thaliana	*AtCBF4*	Improved drought and freezing tolerance (*Arabidopsis*)	Haake et al. (2002)
N. tabacum	*AtP5Cs* and *VacP5Cs* for D 1-pyroline-5-carboxylate synthetase production from *Arabidopsis* or *Vigna SacB for* levansucrase from *Bacillus subtilis* or the *codA* gene coding for CO from *Arthrobacter globiformis*	Accumulation of osmoprotectants like proline fructan or GB	Konstantinova et al. (2002)
L. esculentum	*CBF1*	Freezing tolerant	Hsieh et al. (2002)
		CAT1 activity increased, tolerant to oxidative stress	
A. thaliana	*CBF3*	Freezing tolerant	Gilmour et al. (2000)
		Increase in proline and total soluble sugars	
N. tabacum	*SbwAFP*	Freezing tolerant	Holmberg et al. (2001)
		sbwAFP is targeted to apoplastic space	
A. thaliana	*DREB1A*	Freezing tolerant	Kasuga et al. (1999)
		Induced the expression of multiple COR genes	
N. tabacum	Type II Fish AFP	No difference in frost tolerance from wild type	Kenward et al. (1999)
		Protein found in apoplast	
A. thaliana	*Coda*	Accumulation of GB	Hayashi et al. (1997)
S. tuberosum	Synthetic AFP	Freezing tolerant	Wallis et al. (1997)
		Marginal increase of tolerance	

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Cell Signaling During Drought and Salt Stress

11

Faheema Khan and Khalid Rehman Hakeem

Abstract

Among abiotic factors, salinity and drought stress affect every aspect of plant from physiology to metabolic activities. Understanding of abiotic stress responses and signal transduction to control adaptive pathways is a crucial step in determining the plant resistance exposed to unfavorable environments. Molecular and genomic findings have shown several changes in gene expression profiling under drought and salt stresses in plants. Numbers of transcription factors which are accountable for inducing stress-responsive genes have been documented. To survive in adverse condition, plants have stress-specific and adaptive responses which provide them necessary protection. Although, there are several signaling pathways and stress-responsive perceptions, some of which are definite in function, while others may have cross talk. Expressions of a large number of transcripts and genes are induced by these abiotic stresses in plants which facilitate stress tolerance and stress response. Recently, progress has been made in investigating the complex cascades of gene expression in stress responses. Knowledge about plant stress signaling is essential for the development of transgenic and improving breeding strategies in crops under stress environment. This chapter provides an outline of the common features of stress signaling in plants with some current studies on the functional analysis of signaling machineries under salt and drought stresses.

Keywords

Drought • Salt • Signaling • SOS • ABA • Abiotic stress

F. Khan (✉)
Department of Botany and Microbiology, College of Science, King Saud University, Riyadh 11495, Kingdom of Saudi Arabia
e-mail: drfaheemakhan@gmail.com

K.R. Hakeem
Faculty of Forestry, Universiti Putra Malaysia, Serdang 43400, Selangor, Malaysia
e-mail: kur.hakeem@gmail.com

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11.1 Introduction

In response to human population growth, agriculture faces a constant challenge to increase crop production annually. Throughout the world salinity and drought are responsible for much of the yield decline in agricultural lands. Moreover, constant salinization of arid and semiarid land is becoming more widespread because of poor local irrigation practices, thus decreasing the yield from formerly productive land (Kaya et al. 2010). As land and water resources become limiting, high-yielding crops even in environmentally stressful conditions will be essential. About 6 % of the world's land and 30 % of the world's productive areas are under salinity (UNESCO Water Portal 2007). Further, the rapid change in global climate, which is more than estimated (Intergovernmental Panel on Climate Change 2007), seems to increase dryness for the semiarid regions of the world (Bates et al. 2008; Lehner et al. 2005). Therefore, drought in concert with increasing population will lead to irrational utilization of water resources for crop production. It mostly affects every aspect of the growth and metabolic activity of plants and causes a significant reduction in crop yield (Passioura 2007) equally as salinity does. To avoid abiotic stresses, plant is dependent upon the stimulation of molecular cascades responsible for certain metabolites, stress perception, signal transduction, and controlling expression of stress-responsive genes (Huang et al. 2012). The physiological responses developed against salinity and drought stress were found analogous to each other as both of these stresses eventually lead to osmotic imbalance of the cell. A general response to salt and drought stress as a signaling pathway is shown in Fig. 11.1. High salt and drought stress show their impact on a plant cell by disturbing the osmotic and ionic equilibrium. Excess of Na^+ ions and osmotic imbalance cause changes in turgor pressure, these cellular activities consider as the major triggers of the stress signaling. These preliminary activities lead to a chain of events, which can be further divided as osmotic and ionic signaling pathways leading to stress tolerance. Abiotic stress responses can be noticed by stress in a form of injury such as necrosis and chlorosis. This stress injury causes its deleterious effect on cell division resulting in retarded growth and plant productivity. Shedding of the older leaves is also considered as one of the stress-tolerant response. The possible reasons for the injuries are high Na^+ toxicity, osmotic imbalance, ROS generation, or due to the degradation of plant cellular proteins and enzymes. To avoid stress-related damages, plants activate certain detoxifying processes, which consist of changes in enzymes, chaperones, molecular proteinases, gene synthesis of LEA/dehydrin for ROS scavenging, and other metabolites (Table 11.1). These processes control and restore stress-induced damages and enhance plant stress tolerance. Plant plasma membrane directly or indirectly perceives environmental stress and plays a vital role in the conduction of peripheral signals in plant protection responses to initiate stress signaling pathway. It is reported that physical properties of cell membranes such as lipid and fatty acid composition have the potential to sense external stress (López-Pérez et al. 2009). Rapid advancement of molecular, transgenic, and functional genomics, technologies collectively with the accessibility of cDNA sequencing and gene knockout mutants (Chinnusamy et al. 2004; Yamaguchi-Shinozaki and Shinozaki 2006) have facilitated significant progress in analyzing molecular aspects of the drought and high salinity responses in plants. Many proficient protection responses exist in plants that allow them to perceive and adapt appropriately to a range of stress signal. A number of stress-responsive genes and gene products have been acknowledged against drought and salinity stress. Recently, several hundred genes have been identified that are induced or subdued at the transcriptional as well as proteomic levels when plants or plant parts are subjected to drought and salinity (Mahajan and Tuteja 2005; Miller et al. 2010; Hakeem et al. 2012a, 2013). It has been reported that under multiple stresses, plants elicit distinctive and complex responses regarding respiration, photosynthesis (Mittler 2006; Rizhsky et al. 2002; Hakeem et al. 2012b), and signaling stress (Okamoto et al. 2009). Therefore, the need to

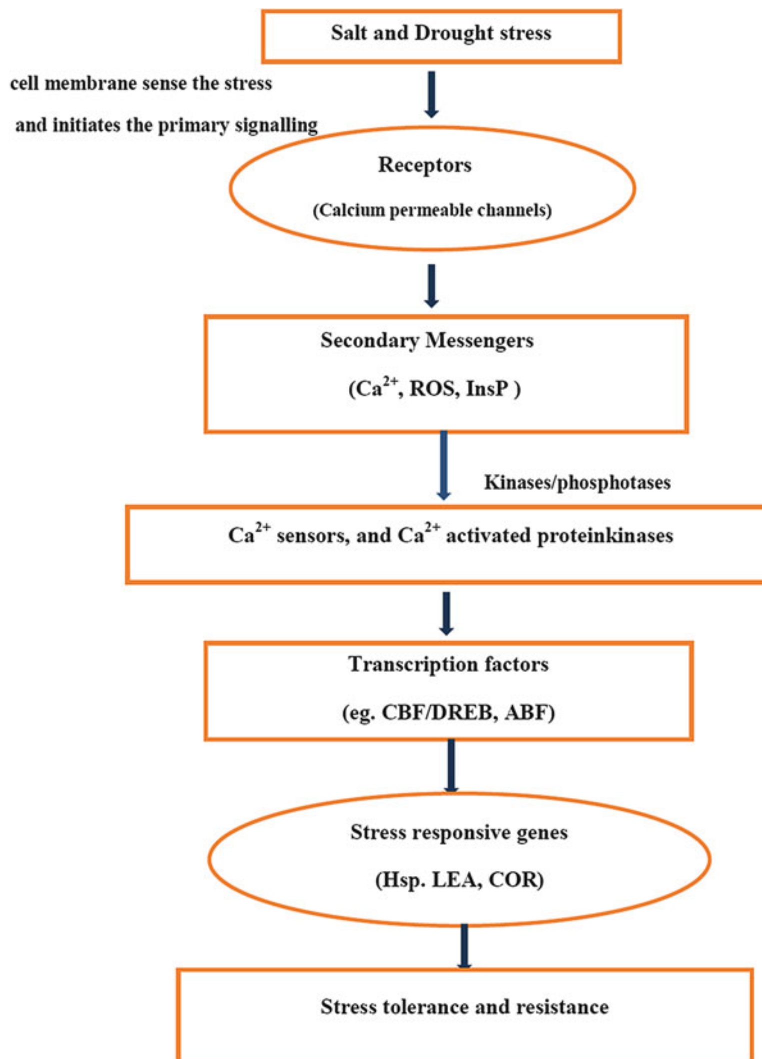

Fig. 11.1 Signaling pathway for plant response to salt and drought stress. The stress signal is sensed by the cell membrane triggering the complex signaling cascade in plants. The signaling transduction induces stress-responsive genes to provide stress resistance

study such interactions under conditions mimicking nature is emphasized (Cimato et al. 2010). Studies showed at molecular level, co-occurrence of different stresses can generate the coactivation of different response signaling pathways (abscisic acid, ethylene, jasmonic acid, etc.) (Mittler 2006). Nature has acquired different protective ways for avoiding and resisting unfavorable conditions, although some of the changes in plants are clearly adaptive, others may have cross talk with each other in response to the stress signals. These signaling pathways act in cooperation to alleviate environmental stress. Knowledge of these pathways is important because they may provide suitable candidate which can be genetically exploit through plant breeding to advance drought and salinity tolerance. In this chapter several abiotic stress signaling pathways are described with the latest research advances in drought and salt stress signaling.

Table 11.1 Response to the salt stress accumulating osmoprotectants and metabolites and there function(s) in conferring tolerance

Product group	Specific compound	Suggested functions	References
Ions	Sodium chloride	Osmotic adjustment	Blumwald et al. (2000)
		Potassium exclusion	Hasegawa et al. (2000)
Protein	Osmotin SOD, catalase	Radical detoxification	Bohnert and Jensen (1996), Allen (1995), Hernandez et al. (2000)
Amino acid	Proline, ectoine	Osmotic adjustment osmoprotectant	Khatkar and Kuhad (2000)
Sugars	Glucose, fructose, sucrose	Osmotic adjustment	Kerepesi and Galiba (2000)
		Carbon storage	Bohnert and Jensen (1996)
Polyols	Mannitol and pinitol	Carbon storage, osmotic adjustment, osmoprotectant Radical scavenging	Bohnert et al. (1995)
Quaternary amines	Glycine betaine	Osmoprotectant	Khan et al. (2002)
Pigments	Carotenoids, anthocyanin, betaines	Protection against photoinhibition	Foyer and Noctor (2005) Adam et al. (1992)

11.2 Stress Signaling Pathways

To study stress adaptive responses, to study adaptive responses, drought and salt signaling transduction can be functionally grouped into three categories: first, ion homeostasis, which is generally significant to salinity and osmotic adjustment; second, controlling stress damage and toxic radical scavenging; and third, to manage cell division and development against stress conditions (Zhu 2001).

11.3 Ion Homeostasis

Homeostasis is defined as the tendency of a cell or an organism to maintain internal steady state in response to any environmental perturbation or stimulus tending to disturb normality. Hypersaline environment, mostly mediated by high level of NaCl, causing perturbation of steady ionic state for NaCl and Cl^- along with K^+ and Ca^{2+} (Niu et al. 1995). Plant cells regulate water relation imbalance through osmotic adjustment by synthesizing compatible organic solutes and ion accumulation. High NaCl concentration causes the disturbance in the steady transport of Na^+ and $C1^-$ ion and other ions, such as Ca^{2+} and K^+ (Hasegawa et al. 2000). High concentration of Na^+ and Cl^- disturbs ionic equilibrium and thermodynamics of plant tissue resulting in ionic imbalance hyperosmotic stress and toxicity. Excess of Na^+ negatively affects intracellular influx of K^+ ion and attenuates the acquisition of this vital element. Intracellular K^+ and Na^+ homeostasis is important for the actions of several cytosolic enzymes and for maintaining appropriate membrane potential across the cell wall. Ion homeostasis is regulated by transmembrane proteins that mediate ion fluxes. It includes H^+-translocating ATPase and pyrophosphatases, Ca^{2+} ATPases, and secondary transporters (Sze et al. 1999). Several transport proteins that mediate K^+, Na^+, and Cl^- transport have been reported on the basis of molecular studied. Dreyer et al. (1999) identified functional complementation of transport-deficient in yeast mutants. Excess NaCl enhances the cytosolic Ca^{2+} accumulation which consequently triggers stress responses. Thus, in salinity and drought it is important for the plant to maintain cellular ion homeostasis for normal growth and metabolic functioning.

11.3.1 Sodium Homeostasis and Ion Compartmentation

High Na^+ in the environment is of pronounced agricultural significance as saline solutions impose both ionic and osmotic stresses on plants. Na^+ ions disturb K^+ uptake by root cells (Hasegawa et al. 2000). Externally Na^+ is sensed by a cell membrane receptor, while intracellular Na^+ is perceived by membrane proteins or either by one, from the several known Na^+-sensitive cytoplasmic enzymes. It has been reported that a plasma membrane Na^+/H^+ antiport activity of *SOS1* (SALT OVERLY SENSITIVE1) stimulates efflux of excess Na^+ ions and maintains Na^+ ion homeostasis (Turkan and Demiral 2009). High Na^+ accumulation in the leaf cell becomes toxic to enzyme production (Hasegawa et al. 2000). Excess of Na^+ results leaf necrosis in older leaves, shortening the life time of individual leaves thus resulting low productivity (Munns 2002). To prevent tissue injuries, plant cell death and growth retardation of unwanted Na^+ has to be removed or follow ion compartmentation (Hasegawa et al. 2000). The pumping of the Na^+ ions in the vacuole involves energy-dependent transportation which alters vacuolar alkalization (Apse et al. 1999). Na^+ compartmentation in vacuole reduces cytosolic levels of Na^+ ions and further facilitates the energetically downhill influx across the plasma membrane. A wide range of membrane transporters assists plants with adaptable approaches to combat external stresses. Phylogenetic study of full genome sequence has shown a large number of putative cation/H^+ antiporters in *Arabidopsis* (Maser et al. 2002). The finest examples of the cation exchangers are the Ca^{2+}/H^+ and Na^+/H^+; they help in retaining the cytosolic concentration of the cell by extruding Ca^{2+} and Na^+, respectively (Hepler 2005). Analogous transport actions are found in plasma membrane and cell organelles (Hepler 2005). In plants the first cation exchanger for Na^+/H^+ was found in *Arabidopsis* (Gaxiola et al. 1999); these exchanger include NHXs, AtNHXl, and AtNHX2 protein. Yokoi et al. (2002) observed the upregulation of these protein exchangers under salt stress. AtNHXNa^+/H^+ antiporters also play a vital role in Na^+ compartmentation (Blumwald et al. 2000). AtNHX1 and AtNHX2 are localized in tonoplast, the expression of these transcripts was found upregulated under osmotic stress and ABA (Yokoi et al. 2002). High salt level increases the transcripts of vacuolar H^+-ATPase components in response to salt tolerance (Dietz et al. 2001). Moreover, AtNHXl also plays an important role in Na^+ storage in the vacuoles of root hair. Since NHX is identified, the number of similar transporters has massively increased. The large number of DNA sequence-encoding NHX-like proteins from more than 60 species including dicot and monocots has been acknowledged in the database of different gene banks.

11.3.2 Ca^{2+} Homeostasis and Secondary Messengers

During abiotic stress the mechanism of signal perception remains unknown and may involve a complex protein-protein interaction and molecule signaling that usually increase or decrease in a temporary, e.g., hormones, ROS, sugars, and Ca^+. Calcium functions as a secondary messenger under various stress conditions and initiates cross talk. Various studies have reported that drought and high salt rapidly raise calcium levels in cells (Pardo 2010). The resulting signaling pathway activates numerous genes that play critical role to sustain cellular homeostasis. Several Ca^{2+} sensors were implicated to be involved in drought signaling. Sequential dynamics of Ca^{2+} transients in response to drought were studied revealing increase in cytosolic Ca^{2+} due to release of Ca^{2+} from the vacuole and cell-type specificity of (Ca^{2+}) transients (Kiegle et al. 2000). Study showed increased intracellular Ca^{2+} levels under salinity stress. Zhu et al. (1998) have identified the determinants of salt tolerance in *Arabidopsis* mutants by applying molecular approach. This approach was helpful in determining the genetic locus which is essential for salt resistance. *Arabidopsis* overexpress the ionotropic glutamate receptor AtGluR2, which decreases the efficiency of Ca^{2+} utilization, those plants become hypersensitive to Na^+ and K^+ ionic stresses (Kim et al. 2001). One of the Ca^{2+} sensor is calcium-dependent protein

kinases (CDPKs) which was reported to be drought induced and the importance of CDPK isoforms in facilitating the stress was demonstrated (Ozturk et al. 2002). Another Ca^{2+} sensor is calmodulin which is Ca^{2+}-binding protein stimulated by increased calcium level. Calmodulin-binding transcription factors were first reported in drought-stressed *Brassica napus* (Bouche et al. 2002). In *Arabidopsis* and rice (*O. sativa*), ABA-activated calmodulins were observed and suggested as harmful regulator of osmotic stress (Perruc et al. 2004). One more Ca^{2+}-binding protein is calcineurin B-like protein (CBLs), and CBL1 was shown to be the only drought-induced CBL (Kudla et al. 1999). The studies have been conducted on annexins which are a family of Ca^{2+}-dependent membrane-binding proteins. It is evident that certain annexins induced cytoplasmic Ca^{2+} under abiotic stress and their upregulation to tolerate osmotic stress is also well recognized. Thus, abiotic stress excites the Ca^{2+} influx from the apoplast and cell vacuoles, thus enhancing the level of cytosolic Ca^{2+}. The Ca^{2+} ions then function as secondary messenger to control K^+/Na^{2+} selectivity and increases K^+ influx (Xiong and Zhu 2001).

11.4 ROS Signaling and Antioxidants

In plants reactive oxygen species (ROS) are constantly produced as a result of different metabolic pathways localized in different cell compartments (Foyer and Harbinson 1994). ROS is a collective term that describes the chemical species that are generated upon incomplete reduction of oxygen. Main ROS molecules are hydroxyl radicals, singlet oxygen, superoxide anion radicals, and hydrogen peroxide. ROS are commonly generated during aerobic period of photorespiration and photosynthesis (Kotchoni et al. 2006). Under abiotic stress increase in these molecules can also be noticed in peroxisomes (Mittler 2002). During physiologically balanced conditions, the ROS molecules are scavenged by various components of antioxidative defense system (Alscher et al. 1997). The balance among generation and scavenging of ROS may be disturbed by a number of environmental conditions which rapidly increases the intracellular levels of these molecules (Tsugane et al. 1999). ROS function as intracellular signaling molecules and control stress damage, as showed by a number of studies. It is investigated that transgenic plants with higher ROS generation or mutants with more ROS-scavenging capacity showed better stress tolerance (Hasegawa et al. 2000; Kocsy et al. 2001). The ROS molecules are extremely reactive and highly energetic compound, they can perform catalytic functions in the lack of cellular enzymes. This property makes ROS molecule appropriate to activate plant stress signaling (Foyer and Noctor 2005). The major electron carriers such as plastoquinone (PQ) or the electron acceptors such as ferredoxin/thioredoxin along with ROS are involved in the redox signaling. To eliminate ROS, plants intrinsically develop different types of antioxidants which can reduce oxidative damage and confer drought and salt tolerance. The ROS-scavenging mechanisms comprise of enzymatic and nonenzymatic molecules. Antioxidative enzymes such as SOD, CAT, POX, GR, and APX are produced in subcellular organelles with a highly oxidizing metabolic activity such as mitochondria, chloroplasts, and peroxisomes to overcome ROS toxicity (Mittler 2006; Foyer and Noctor 2003; Miller et al. 2008; Ahmad et al. 2012). ABA accumulation induced by drought stress activates the generation of ROS, which leads to the upregulation of the antioxidant defense mechanism in plants (Hu et al. 2005). Free radical-mediated lipid peroxidation (MDA) results in highly reactive and toxic aldehydes, which are scavenged by either aldehyde dehydrogenases or aldehyde reductases. There are several studies evident of the involvement of these enzymes in drought stress response (Sunkar et al. 2003). ROS are known as second messengers in redox signaling and also involved in hormonal-mediated actions (Foyer and Noctor 2003). It has been reported that ABA induces the gene expression of some antioxidant enzymes such as CAT, SOD, and APX (Park et al. 2004) and also enhances the activities of these enzymes in cell (Zhang 2003). Recently, it was demonstrated that changes in the auxin substrate of a

H_2O_2-responsive enzyme involved in anthocyanin production facilitate drought resistance (Tognetti et al. 2010). In another study, mutants with decrease anthocyanin levels were more drought tolerant (Huang et al. 2010). These data support a role of anthocyanins, which are antioxidants, in drought tolerance along with ROS and phytohormones. The implicated molecules include glutathione peroxidase and cellulase-synthase-like protein (Zhu et al. 2010). Moreover, squalene epoxidase implicated in sterol biosynthesis was also reported to have a role in the localization of NADPH oxidases essential for regulation of ROS (Pose et al. 2009). Under high salt environment, regulation of NaCl responses is controlled by ROS. In *Arabidopsis* cells NaCl stress comprising both ionic and osmotic stresses has recently exhibit to induce development of endosomes having high level of hydrogen peroxide (Leshem et al. 2007). ROS production has been triggered within endosomes of *Arabidopsis* root cells (Leshem et al. 2007). The recent studies also suggest new vital regulatory roles of ROS in intracellular trafficking through vesicles in addition to their role in retrograde stress signaling in plants under abiotic stress (Miller et al. 2010). ROS-scavenging enzymes have been involved in signaling as well as their more customary role in protection from oxidative stress in recent years (Miller et al. 2010). In transgenic plants overproduction of antioxidant enzymes has improved drought and salt tolerance (Eltayeb et al. 2007; Lu et al. 2007; Tseng et al. 2007). It seems that integrated signaling networks are accountable for the stimulation of transduction pathways. Moreover, it has been also reported that while some changes in ROS metabolism enhanced resistance to stress, other changes caused enhanced sensitivity (Miller et al. 2010).

11.5 SOS Regulatory Pathway

To combat abiotic stresses, numbers of signaling pathways have been suggested, although not any is recognized in terms of signaling proteins inputs and outputs. Recently SOS pathway emerged as an exception from the result of biochemical, genetic, and molecular analysis (Zhu 2001). Gene analysis associated in salt resistance was commenced in 1998, by Liu and Zhu where numbers of mutants were checked and through cloning SOS (salt overly sensitive) genes were identified. SOS pathway helps in eliminating excess Na^+ ions via plasma membrane and Na^+/H^+ antiporter which assist in maintaining ion homeostasis in the cell. Identification of SOS genes has broadened the way for revelation of a unique pathway associated with Ca^{2+} signaling in response to salt fluctuations (Liu et al. 1998). SOS pathway consists of a calcium-binding protein known as SOS3 which senses cytosolic fluctuations in calcium, stimulated by high salt. SOS3 also activates the protein kinase, SOS2. SOS1, SOS3/SOS2 kinase complex activates together and phosphorylates the transport action of the plasma membrane. Besides the transport function, SOS1 may also play a vital role in regulating and sensing Na^+ ions. SOS2 gene was isolated from *Arabidopsis* by the genetic selection of hypersensitive salt mutants. High salt concentration showed elevated SOS2 transcripts in response to stress tolerance in *Arabidopsis* roots (Liu et al. 2000). Moreover, SOS2 interacts with vacuolar Na^+/H^+ antiporter and considerably controls the cationic (Na^+/H^+) exchange activity (Qiu et al. 2002).

11.6 Protein Kinase Pathways for Osmotic Stress Signaling

The role of protein phosphorylation in response to osmotic has been proved experimentally (Zhu 2001). Many protein kinases in plants have been found activated in response to osmotic stress. The illustrated osmosensing pathway found in yeast (Gustin et al. 1988) created much attention to investigate a similar pathway in plants. In yeast the osmoregulatory pathways initiate with SH3 domain having membrane proteins which further activate MAP kinase cascade and elevate the osmolyte accumulation in the cell (Gustin et al. 1988). Under osmotic stress the plant synthesizes and accumulates compatible solutes for cellular adjustment. For synthesizing osmolyte it is indistinct whether they utilize similar

membrane sensors and MAP kinase cascades. In plants several MAPKs (mitogen-activated protein kinase) are stimulated against osmotic stress. MAP kinase (46 kDa) named SIMK (salt stress-inducible MAPK) found increased in response to adequate osmotic stress in alfalfa (Munnik et al. 1999). Hyperosmotic stress activated a SIMK-like MAP kinase named SIPK (salicylic acid-induced protein kinase) in tobacco cells (Mikolajczyk et al. 2000). MAPK cascade includes three protein kinases (MAPK, MAPKK, and MAPKKK) which are activated by serial phosphorylation. They result in specific localization of the module in cell compartments, phosphorylation, and regulating transcription factors and other proteins. A number of MAP kinases were identified using sequence information in *Arabidopsis* (Ichimura et al. 2002). Interestingly, during hyperosmotic stress SIMK was not stimulated and as an alternative a smaller kinase got triggered which suggest that the two kinases activated at different salt concentrations. Taking into consideration the different studies showed that one MAP kinase can respond to different stress conditions and there are different numbers of proposed and identified MAP kinases. From each of the three categories, there should be a convergence in the signaling of MAPK cascade, and it is possible that different stress factors activate MAP kinases to different levels (Bartels and Sunkar 2005). SNF-1-like kinases, divided into three families, SnRK1, SnRK2, and SnRK3, are another family of protein kinases which are activated by the phosphorylation of their serine or threonines (Halford and Hardie 1998). In various plant species, several SNF-1-like kinases were predicted and shown to be expressed in response to dehydration or ABA, including *Arabidopsis* OPEN STOMATA1 (OST1) protein kinase (Bartels and Sunkar 2005).

11.7 Phospholipid Signaling

In plants phospholipid membrane plays an important structural role. Environmental stress creates a dynamic system that produces a number of signal molecules, e.g., PA, IP3, and DAG. The signaling of phospholipids is grouped on the basis of phospholipases that catalyzes lipid production. Studies suggest that some novel signaling pathways are involved in the production of lipid messengers which are not the direct yields of phospholipases. Examples of such lipid messengers are phosphatidylinositol 3,5-bisphosphate [PI(3,5)P2] and diacylglycerol pyrophosphate (DGPP) (Munnik 2001). Acting as a secondary messenger, phospholipase C (PLC) catalyzes the hydrolysis of phosphatidylinositol 4, 5-bisphosphate (PIP2) into IP3 and DAG. Under hyperosmotic stress condition, the level of IP3 found increased (DeWald et al. 2001). It is observed that osmotic stress enhances the activity of phospholipase D (PLD) in tomato and alfalfa (Frank et al. 2000). Phospholipase D found activated under drought stress in *Craterostigma plantagineum* and *Arabidopsis* (Katagiri et al. 2001). Plant PLD activity was analyzed in drought-tolerant and drought-sensitive cowpea cultivars; higher PLD activity was found in the drought-sensitive cultivars (Maarouf et al. 1999). In response to hyperosmotic stress, various plant systems showed rapid increase of 1,4,5-trisphosphate (IP3) (Takahashi et al. 2001; DeWald et al. 2001). According to current hypothesis drought activation of PLC leads to higher IP3 levels. A subsequent pH release of Ca^{2+} to cytoplasm and triggering of K^{+} ion channels result in stomatal closure (Takahashi et al. 2001). In *Arabidopsis* SAL1 belongs to the latter group, and recently studies have supported drought-tolerant SAL1 mutants as a negative regulator in drought signaling pathways (Wilson et al. 2009). In another recent study, an inositol phosphate-lacking transgenic plant was generated by the expression of inositol polyphosphate 5-phosphatase (Perera et al. 2008) which showed higher drought resistance. Several plant species showed increase PLDs in response to induced ABA and drought stress (El-Maarouf et al. 2001; Frank et al. 2000; Katagiri et al. 2001).

11.8 ABA and Osmotic Stress Signaling

In plants ABA is needed for various stress responses, comprising stomatal closure, metabolic changes, and stress-responsive gene regulation. During unfavorable conditions the endogenous

ABA plays a vital role in stress-induced ABA-dependent activities. ABA level varies widely in response to environmental stress, especially under drought and salinity. The level of ABA synthesis and catabolic activities measured the level of endogenous ABA. Lately, the molecular source of ABA biosynthesis was determined by genetic and biochemical approaches (Nambara and Marion-Poll 2005). Another precursor of ABA is xanthoxin which results from a direct cleavage of C40 carotenoids; this reaction is considered as a vital step in ABA biosynthesis. ABA is an important phytohormone and has wide range of action including plant growth and development. One of the main functions of ABA is to regulate osmotic stress plant maintain water balance. This role can be best justified by plant mutants that are unable to synthesize ABA. In *Arabidopsis* a number of ABA deficient mutants have been identified such as aba1, aba2, and aba3 (Koornneef et al. 1998). ABA produced in the roots is further carried to the shoots which cause the stomatal closure and ultimately restricts cellular growth of the plant (Wilkinson and Davies 2002). Drought and high salinity might cause higher pH in xylem sap (Jia and Davies 2007), resulting in modulation of stomata in response to abiotic stresses. Several genes which can synthesize ABA have now been reported, e.g., in tobacco zeathanxin epoxidase known as ABA2 and in *Arabidopsis* ABA1: they catalyzes the zeaxanthin and antheraxanthin to violaxanthin (Marin et al. 1996). It has been studied that induced ABA stomatal closure is moderately dependant on activity of NADPH oxidase (Kwak et al. 2003; Torres and Dangl 2005). It has been studied that osmotic stress caused by salinity or drought is conducted via two pathways. One is dependent on ABA and the other is ABA independent; genetic investigation found no clear difference between ABA-dependent and ABA-nondependent pathways, and therefore, the mechanisms of this signaling may often have cross talks (Xiong and Zhu 2001). Ca^{2+} which is one of the essential elements and conferred to act as a second messenger for several stress responses mediates such cross talk. Studies have proved that high salt, drought, and ABA increase intracellular calcium in plants (Sanders et al. 2002).

11.9 Transcriptional Signaling

Transcriptome profiling has acknowledged number of gene-encoding transcription factors that are either upregulated or downregulated by multiple stresses. The expression of these transcription factors provides us deeper insight in understanding the stress tolerance mechanism. Cheng et al. (2002) reported numbers of stress-induced transcription factors for biotic and abiotic stresses in *Arabidopsis*. Among this group approximately 20 genes were found upregulated in response to osmotic stress and salinity. Recently, gene expression profiling using gene chips and cDNA microarrays has identified number of potential genes that are either upregulated or downregulated under drought and salinity (Bohnert and Cushman 2001; Seki et al. 2001). Alterations in gene expression, in response to drought and salinity, varies to the type and degree of stress. *Arabidopsis* provided profound insights of functional genomics related to multiple stress response. Using 1,300 full-length clones (Seki et al. 2001) and 7,000 full-length clone inserts (Seki et al. 2002), multi-stress interactions in response to abiotic stress were studied. The study provided potential stress-induced genes in response to salinity and drought stress. To study abiotic stress treatments, multi-stress interactions were studied by Kreps et al. (2002) in *Arabidopsis*. The study provided the alterations in gene expression more than twofold over control for 2,409 out of 8,100 genes in response to drought and high salt. Under drought stress in chickpea roots, transcripts were found twofold (STCa-8875; arginine decarboxylase) and threefold (STCa-611; spermidine synthase) upregulated (Molina et al. 2008). The study showed a different influence of drought stress on mechanisms of osmolyte accumulation as an early stress response in chickpea roots. Numbers of transcription factors which belong to the EREBP/AP2 family bind to DRE/CRT were identified and group as CBF1/DREB1B, CBF2/DREBC, and CBF3/DREB1A (Liu et al. 1998). Analogous transcription factors DREB2A and DREB2B that are induced under hyperosmotic condition were also reported as stress-responsive genes (Liu et al. 1998).

11.10 Conclusion and Perspectives

Salinity and drought stress signal transduction has remained a major agriculture ambiguity until now and regarded as complex network. Recently, molecular biology has paved a way in identifying some stress-related signaling element which widens the knowledge of plant stress response. There are several views of convergence and divergence that facilitate stress signaling cascades. From this chapter, we know that to overcome drought and salinity, plants have stress-specific signaling pathways. Plant stress conditions enhance the production of ROS along with ABA accumulation, which has been suggested to be key constituents of cross tolerance to multiple types of stresses. The oxidative stress was used to be considered as a negative activity, but now observed as fundamental stress response which induces tolerance mechanism. Signaling pathway such as SOS found to play a critical function in regulating osmotic stress, whereas there are cross talks between MAPK cascade and ABA signaling pathway. Though latest progresses have acknowledged numerous transduction factors involved in stress tolerance, there is an urgent need to fill the gaps through wider application genetic engineering in model plants. With the emerging powerful tools such as genomics, transcriptomics, and proteomics, advancement in understanding plant stress signaling will positively increase and may provide better insight of stress tolerance mechanism.

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Heat Signaling and Stress Responses in Photosynthesis

12

Marian Brestic, Marek Zivcak, Katarina Olsovska,
Hazem Mohamed Kalaji, Hongbo Shao,
and Khalid Rehman Hakeem

Abstract

High temperature represents one of the most serious abiotic stress factors limiting plant photosynthesis, biomass production, and crop productivity. Photosynthetic apparatus is an important heat sensor in plants, sensing a wide range of air temperatures, from moderate to extreme. In this chapter we offer current knowledge on both photochemical and metabolic changes occurring within the photosynthetic apparatus in conditions of heat stress associated with signaling and stress response. The heat stress directly affects the heat-sensitive sites, mainly oxygen-evolving complex of photosystem II and Rubisco activase. It leads to subsequent indirect effects, such as changes of the redox status of individual components on thylakoid membrane in chloroplast and increase in production of reactive oxygen species (ROS). Hence, the redox signaling plays the crucial role in enhancement of alternative electron pathways such as cyclic electron flow as well as triggering the signal transduction pathways resulting to heat-stress response. The redox signaling in chloroplast is closely associated with ROS signaling, which interferes with regulation also out of chloroplast. The stress response involves mainly production of specific proteins (mostly heat shock proteins or antioxidants) or protective

M. Brestic (✉) • M. Zivcak • K. Olsovska
Department of Plant Physiology, Slovak Agricultural University, Tr. A. Hlinku 2, 949 76 Nitra, Slovak Republic
e-mail: marian.brestic@uniag.sk

H.M. Kalaji
Department of Plant Physiology, Faculty of Agriculture and Biology, Warsaw Agricultural University SGGW, Nowoursynowska 159, 02-776 Warsaw, Poland

H. Shao
Institute of Life Sciences Qingdao, University of Science &Technology, Zhengzhou Road 53, Qingdao 266042, China

Yantai Institute of Coastal Zone Research, Chinese Academy of Sciences, Chunhui Rd.17, Yantai 264003, China

K.R. Hakeem
Faculty of Forestry, Universiti Putra Malaysia, Serdang 43400, Selangor, Malaysia
e-mail: kur.hakeem@gmail.com

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compounds (osmoprotectants) leading to increase of thermostability of sensitive sites or protection against ROS. Different signal molecules contribute in photosynthesis-related heat-stress signaling pathways, such as reactive oxygen species with hydrogen peroxide, nitric oxide, calcium, and abscisic acid. The specific roles of cytokinins and isoprene in heat-stress response are also reviewed.

Keywords
Heat stress • Photosynthesis • Photosystem II • Stress signaling • Reactive oxygen species

12.1 Introduction

Plants perform optimally only in a certain temperature range, from quite narrow to a wide range of temperatures, depending on their species thermo-sensitivity. Increasing temperatures can affect different vital functions of cells and whole plants, such as enzyme activity, membrane integrity, cell division, photosynthetic reactions, growth, and productivity. As plants represent the sessile organisms, which cannot escape efficiently from heat stress, their metabolism and physiology can adapt to counterbalance this disadvantage (Mittler et al. 2012). Often they acclimate to heat in the course of their life cycle if pretreated by moderate heat stress, which enhances their thermotolerance. As a part of this acclimation response, both increased leaf transpiration and oxidative stress take place in heated leaves and cells. Transpirational cooling helps to balance energy surplus and protect leaf tissues against the damage from heat without spending too much ATP energy, but the mechanism can only be effective if plants have enough water in the soil. Because high temperature is often accompanied by low water availability in soils, this may cause leaf water potential decrease amplifying the negative effect of heat stress. In conditions of extreme, even short-time heat, plant tissues are directly exposed to heat and undergo irreversible cell collapsing.

In this chapter we will focus mostly on studies about photosynthetic responses of plants to increase ambient temperature, particularly on heat signaling, signal molecules, and pathways associated with heat-stress response.

12.2 The Significance of Temperature in Plants

As immovable, higher plants need the ability to sense the short- and long-term fluctuations in temperature and to realize the efficient physiological response by active adjustment of their biological functions to efficient functioning in the subsequent temperature conditions (Browse and Xin 2001). The universal mechanism of physiological or photosynthetic response to temperature, which would be valid for all crop species, cannot be described. Plants respond to changes in ambient temperature rather than to absolute temperature. Moreover, different species have inherent genetic diversity and different strategy in growth and development. Thus, the term “high temperature” is a relative term and will be different for psychrophilic, mesophilic, or thermophilic organisms and also for C3 and C4 plants (Falk et al. 2004).

Supraoptimal temperature conditions lead to complex, still not fully understood effects on integrative plant physiology and result to heat stress. It significantly impairs the cellular homeostasis both at the level of membrane and protein stability. To minimize or avoid the negative heat-stress effects, plants need to trigger appropriate responses (Yeh et al. 2012).

Transduction of the high temperature signals seems to be realized by nonoverlapping and independent pathway components (Sung et al. 2003). The perception and transduction of heat-stress signals result in changes at the molecular level, namely, the expression of genes and production of transcription factors, leading in the next step to synthesis of stress-related proteins and enhancement of stress tolerance. It is well known that especially the expression of specific heat shock proteins (HSPs) represents an important adaptive strategy. The high temperature resistance provided by HSPs improves the functions of the main physiological processes, especially photosynthesis, growth, assimilates partitioning, maintaining plant-water relations, etc. Hence, plant growth and development can be maintained under heat stress. However, it is clearly evident that not all plant species or genotypes dispose with similar capacity to cope with the high temperature stress. There is huge interspecific and intraspecific variation providing thus opportunities for improvement of heat-stress tolerance in crop plants (Wahid et al. 2007).

12.3 Heat Sensing and the Primary Component of Heat-Stress Signal Transduction Pathway Network in Plants

The improvement of crop tolerance and productivity under high temperature stress calls for a deeper understanding of the problem of heat sensing and heat-stress responding in plants. In plant cells, there is no specific molecule playing the role of thermosensor. However, there are various intracellular signaling molecules that resulted from the exposure of plants to external heat, that are integrated into the multiple signal transduction pathway network involving different cell compartments as well as cells allowing either acclimation response in a case of moderate heat (warming) or dying as a result of extreme heat (Samach and Wigge 2005). The aim of the network is to regulate gene expression followed by changes in plant transcriptome, proteome, and metabolome enabling plants to acclimate for surviving in the response of heat (Fig. 12.1).

There are many evidences that in plants responding to heat, two principal elements operate on the signal transduction pathway network, such as intracellular calcium (Ca^{2+}) and protein kinases, enzymes which phosphorylate and, hence, increase activity of different target proteins. Together, the signaling pathways that utilize Ca^{2+} ions and protein kinases constitute a network of great complexity and importance (Buchanan et al. 2000).

The primary sensor of a temperature change is plasma membrane of plant cells. Fluidity of membranes is given mainly by the lipid composition, the membrane lipid saturation, and temperature of the environment. Change in membrane fluidity caused by high temperature occurs immediately when plants are exposed to temperature stresses. It represents probably a site of stress signal perception and/or injury (Jan et al. 2009).

As a result of even moderate temperature increases not exceeding the temperature optima for growth, both composition and physical state of plasma membrane change, which in turn, influence the membrane fluidity and, consequently, controlled passage of calcium ions across the membrane (Saidi et al. 2009). Specifically, the level of linoleic-like polyunsaturated fatty acids of the plasma membrane decreases, and conversely, the proportion of oleic acid and other mono- and diunsaturated fatty acid increases, leading to an increase of the membrane fluidity and transient opening of specific Ca^{2+} channels (Saidi et al. 2010). There is still very little information on the nature of these channels; however, what we already know is that Ca^{2+} ions passing through the channels bind to calmodulin-binding domains that activate at least two specific calmodulin- and kinase-dependent signaling pathways. Generally, these pathways result in an upregulation of the heat shock protein (HSP) expression in different compartments of heated cells (Ruelland and Zachowski 2010). Some authors demonstrate also a heat-induced protein denaturation (unfolding) as another important component of the heat-stress response in plants, explaining that it can play a signaling role in

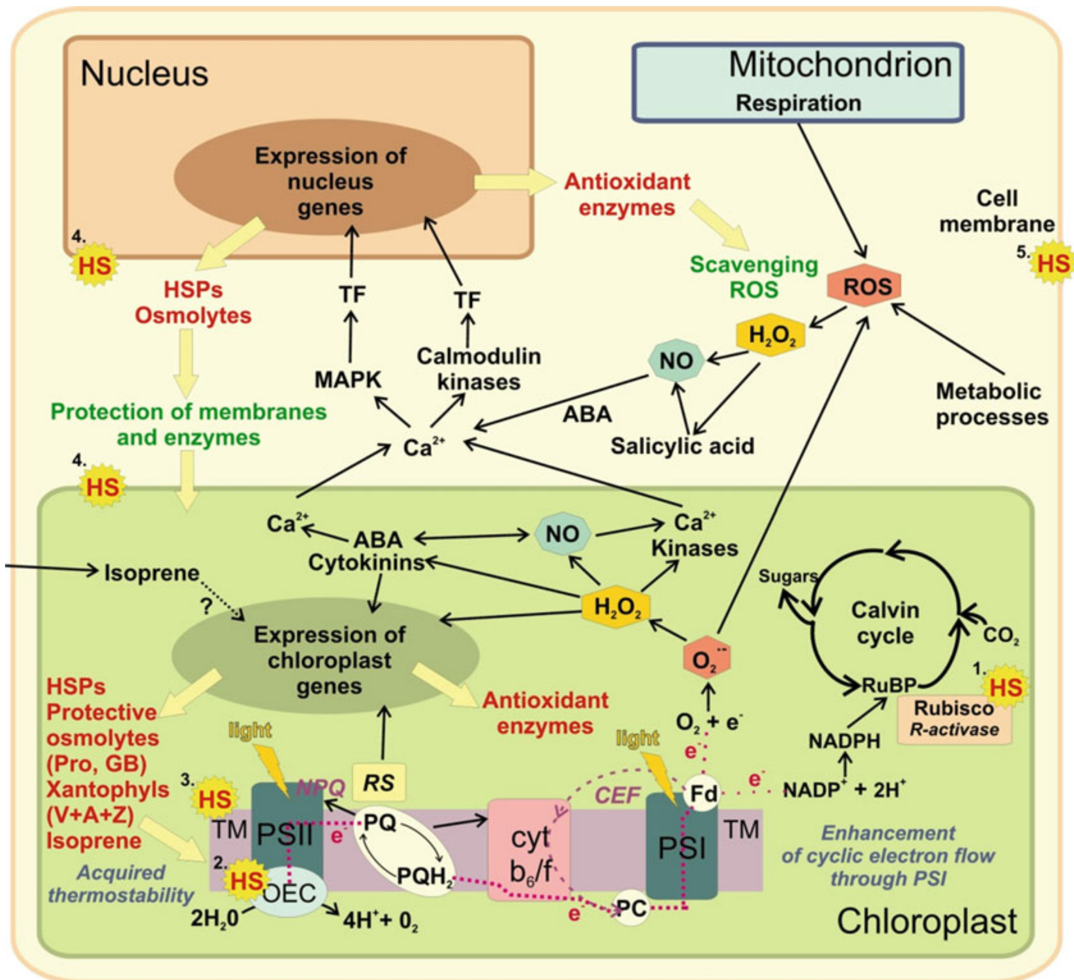

Fig. 12.1 The pathways of photosynthesis-related signal transduction in heat stress. In high temperature conditions, the Rubisco activation declines, resulting to lower utilization of electrons for NADP reduction. The excess of electrons produced by linear electron transport leads to reduction of molecular oxygen, producing the superoxide anion radicals $O_2^{\bullet-}$ at PSI, which are converted into hydrogen peroxide in the reaction catalyzed by SOD. H_2O_2, in addition to its harmful effects, acts as a signal molecule within a signal cascade containing also nitric oxide (NO), abscisic acid (ABA), protein kinases, and calcium cation Ca^{2+}. The pathways can contain also other components, such as salicylic acid and cytokinins. The result of the signal cascade can be the expression of genes in chloroplast resulting to stress response. Moreover, the ROS molecules as well as other signal molecules (mainly Ca^{2+}) can move from chloroplast to cytoplasm, where they contribute to signal transduction pathways, resulting to expression of stress-inducible genes in nucleus. Both nucleus and chloroplast gene expression lead to stress responses: 1. enhancement of antioxidative system protecting against oxidative damages caused by ROS as well as 2. synthesis of protective compounds like heat shock proteins (HSPs), osmolytes (e.g., proline (Pro), glycine-betaine (GB)), xanthophylls of xanthophyll cycle (violaxanthin, antheraxanthin, and zeaxanthin, V+A+Z), and in some species also volatile compounds like isoprene. The protective compounds increase the thermostability of heat-sensitive sites (HS) within plant cell and chloroplast, which are mainly *1*. Rubisco activase and other enzymes, *2*. oxygen-evolving complex (OEC), *3*. thylakoid membrane (TM), *4*. other membranes in organelles, and *5*. cell membrane. In addition, the pathway of redox signaling based on sensing of plastoquinone (PQ) redox status is included. The redox sensor (RS), for example, thioredoxin, can regulate expression of plastid genes, similarly to previously mentioned pathways. Moreover, the PQ redox status regulates also the electron transport rate through cytochrome b6/f avoiding the overexcitation of PSI acceptor side and producing the excess of ROS. The excess of light on PSI is utilized by cyclic electron flow (CEF); on PSII is the excess of energy dissipated in the process of non-photochemical quenching (NPQ). The scheme was built on the basis of recent papers (Hung et al. 2005; Pfannschmidt et al. 2009; Saidi et al. 2011; Kreslavski et al. 2012; Munné-Bosch et al. 2013)

activation of some heat-stress transcription factors (Yamada et al. 2007; Sugio et al. 2009).

Inactivation of these proteins is due to direct or indirect conformational changes that cause either switching their biological function off (Sangwan and Dhindsa 2002) or even changing their biological function as a result of their oligomerization following the conformational changes (Park et al. 2009). The transcription factors upregulated from the heat-stress signal transduction pathways represent only a little part of the total plant transcriptome (5 %), and only a small part of the transcript encodes heat-induced molecular chaperones (Vierling 1991; Larkindale et al. 2005; Richter et al. 2010). Plant chaperones, mainly that belonging to the Hsp70 and Hsp90 families, are small proteins known to prevent cell misfolding of proteins and their aggregation, and thus they protect cell membranes in conditions of heat stress. Except of their role in maintaining the protein homeostasis and membrane integrity, they also operate as negative feedback regulators of the heat-stress response (Hahn et al. 2011).

12.4 Photosynthesis as a Heat Sensor

Within the photosynthetic pathways, the photochemical as well as biochemical processes are integrated. In this respect, temperature conditions will influence the photosynthesis through its indirect or direct effects on different thermally sensitive sites and processes, such as the electron transport chain, photosynthetic carbon reduction, synthesis of sucrose, and carbon partitioning (Falk et al. 2004). Among all metabolic processes in plant cell, the photosynthetic activity in chloroplasts refers to the most heat-sensitive processes (Berry and Bjorkman 1980; Yordanov et al. 1986). It means that photosynthesis either can tolerate moderately high temperatures due to reversible changes in the photosynthetic apparatus or can be damaged as a result of severe heat.

Photosystem II (PSII) localized in the thylakoid membrane of chloroplast has been considered for many years as the primary site of high temperature impairments of the photosynthetic function. PSII was found to be the thermolabile, whereas photosystem I (PSI) has been shown as a heat-resistant component of the photosynthetic electron transport chain. On the thylakoid membrane occur also reversible heat-induced changes, such as a large shift of redox status of PSII and PSI, where PSII becomes more oxidized and PSI becomes more reduced (Datko et al. 2008). High temperature-induced deactivation of PSII involves impairment of the oxygen-evolving complex (OEC) as well as separation of pigments of peripheral light-harvesting complex unit (LHCII) from the PSII complexes (Sharkey and Zhang 2010). During heat stress both the *Chl* $a+b$ content and *Chl/carotenoid* ratio decrease, and the *Chl a/b* ratio increases. The rise of Chl a/b ratio results from faster degradation of chlorophyll *b*, and it indicates a major degradation of light-harvesting chlorophyll-binding proteins associated with PSII (LHCII). Hence, this leads to decrease in the transfer of excitation energy to the PSII reaction center. However, this decrease can be considered as protective mechanism, as this process can reduce the risk of photooxidative damage by decreasing absorption of the light energy by photosystems (Špundová et al. 2003). High temperature stress led to structural and functional disorder of the photosynthetic apparatus and damage to the PSII, when a significant part of the PSII reaction center becomes inactivated. The most efficient tools for monitoring heat effects on PSII are based on the chlorophyll fluorescence recording (Brestic and Zivcak 2013). The PSII heat susceptibility depends on plant species and genotype as well as on the acclimation status of individual plant (Fig. 12.2).

The accelerated PSI activity at high temperature is associated with increased cyclic electron flow around PSI. One or more pathways of cyclic electron transport can be stimulated, and it can lead to an increase or maintenance of the gradient of energy across the thylakoid membranes without increase of NADPH production (Shikanai 2007; Rumeau et al. 2007). Damage of the oxygen-evolving complex (OEC) within PSII and/or the destabilization of antennae complexes (mainly LHCII) on PSII will lead to decrease of NADPH supply for CO_2 fixation and decrease in light activation of the key regulatory enzymes of the Calvin cycle by the thioredoxin (Georgieva 1999).

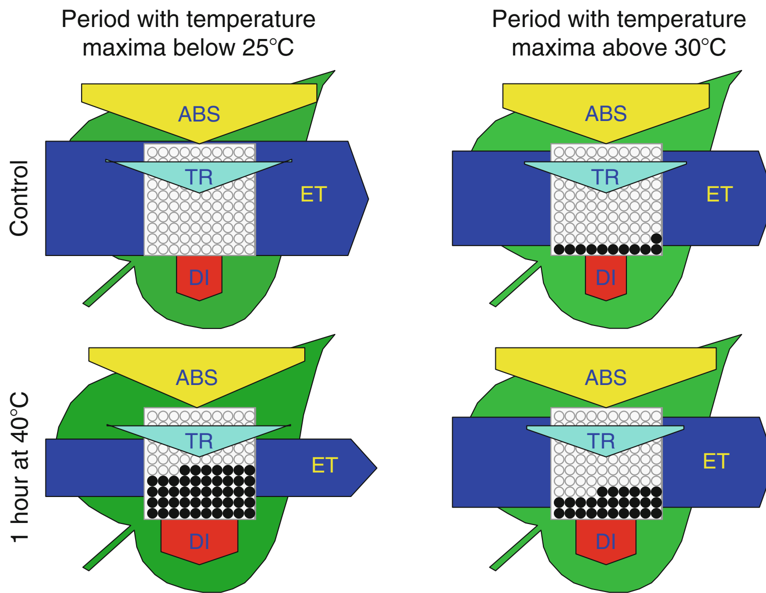

Fig. 12.2 Example of environmentally induced increase of PSII heat thermostability, demonstrated by phenomenological leaf models based on parameters calculated by equations of Strasser et al. (2000) for control and heat-stressed wheat leaves taken from field-grown plants of winter wheat before high temperature occurred and in period with high temperature over 30 °C. The thickness of each arrow represents the value of absorbance (*ABS*), trapping flux (*TR*), electron transport (*ET*), or heat dissipation of excess light (*DI*). The *black points* represent the fraction of inactive reaction centers. Measurements were done by fluorometer Handy PEA (Hansatech, England) and the models were generated using software Biolyzer 3.06 (Maldonado-Rodriguez, Laboratory of Bioenergetics, University of Geneva, Switzerland). Heat stress was induced by exposure of leaf segments to high temperature for 1 h in the dark (Original figure based on the data published by Brestic et al. 2012)

Heat tolerance of cyclic electron flow is longtime known as a mechanism of heat tolerance. If the thylakoid membrane becomes leaky as the result of heat stress, the cyclic electron transport keeps the sufficient supply of ATP (Bukhov et al. 1999). The ATP levels do not decrease at heat stress, even if thylakoid processes are strongly impaired (Schrader et al. 2004). The heat-induced changes of thylakoid membrane are associated also with integrity of the thylakoid membrane and increase of lipid fluidity, which affect the structural stability of thylakoid membrane protein complexes (Godde and Bornman 2004). These changes are accompanied by increased cyclic electron transport, which is normally rather small; however, under high temperature it increases considerably; thereby ATP homeostasis and energy gradient across the thylakoid membrane are maintained. Otherwise, plants suffer from irreversible damage of thylakoid structure and losses of their component functions.

The stress caused by supraoptimal temperature leads also to state 1 to state 2 transitions. This process ensures preferential transfer of excitation energy in favor of PSI. Moreover, the deep oxidation of xanthophylls in PSII antennae (violaxanthin to antheraxanthin and zeaxanthin) increases tolerance of PSII and thylakoid membranes to heat stress by enhancement of non-photochemical quenching of excessive light energy (Havaux and Tardy 1996).

At high temperatures (35–45 °C), the degradation of protein accelerates, but the synthesis of proteins decreases; however, this is not valid for family of heat shock proteins (HSP), which should protect the key protein complexes of the electron transport chain (Coleman and McConnaughay 1994). Mainly HSPs with low molecular weight (HSP21 and HSP24) are transported to the chloroplast from the cytoplasm in high temperature conditions (Godde and Bornman 2004).

According to Waters et al. (1996), several heat shock proteins play a role of molecular chaperones. They refold previously denatured proteins or they can have also a proteolytic function.

It was documented that activation of Rubisco by light is inhibited even in conditions of moderate high temperatures. Such inhibition correlated with reversible decrease of CO_2 assimilation. Moreover, the quantum yield of CO_2 assimilation is decreased also due to increase of photorespiration, which can serve as photoprotective mechanical, as at high temperatures the fraction of the total absorbed energy dissipated by high-energy-state quenching of excitation energy decreases (Falk et al. 2004). According to Feller et al. (1998), the decreased assimilation of CO_2 is caused mainly by the inhibition of Rubisco activation via a direct effect on Rubisco activase, which triggers the catalytic activity of enzyme Rubisco. In addition to previously mentioned mechanisms, the capacity of the primary photosynthetic processes can be limited also by feedback inhibition due to reduced carbon metabolism at high temperatures (Falk et al. 2004).

In nature, plant responses to fluctuations of temperature are modified by many other co-occurring environmental factors to which the plants are exposed. High temperature may be accompanied, e.g., by water deficit and/or excessive light. It was shown that drought can lead to enhancement of heat tolerance of PSII; similarly, expositions of plants at supraoptimal temperature lead to enhancement of PSII. The response of photosynthesis to high temperature depends strongly on species but also on the developmental history of individual plants or leaves. Stomatal conductance appears to play only a minor role in the temperature limitation of photosynthesis (Godde and Bornman 2004).

12.5 Signaling Pathways and Specific Molecules in Heat-Stress Response

Heat-stress response (HSR) is very complex and there are many links between different pathways. Many of them partially or fully act in chloroplast, or they are linked to photosynthetic function. The importance of different heat shock response pathways has been examined on different models, like mutants or transgenic plants with limited function of particular signal pathways (Larkindale and Knight 2002). There are many signal molecules playing important role in high temperature response (included in Fig. 12.1 above), as documented by numerous studies in different species (Table 12.1). The roles of some specific signal molecules or pathways in heat-stress response are presented in the following subchapters.

12.5.1 ROS Signaling Pathway

Plant stress leads in plant cells to the excessive production of reactive oxygen species, mainly hydroxyl radical (HO•), superoxide radical ($O_2^{\bullet-}$), and hydrogen peroxide (Wise and Naylor 1987). Molecules of ROS are very reactive attacking the DNA, membranes, or proteins in cells (Apel and Hirt 2004). Plants scavenge ROS under stress conditions both with nonenzymatic and enzymatic pathways. Enzymes, including ascorbate peroxidase (APX), catalase (CAT), superoxide dismutase (SOD), peroxidase (POX), and glutathione reductase (GR) (Mittler 2002), and nonenzymatic antioxidants such as ascorbic acid (AsA), glutathione (GSH), and tocopherols (Noctor and Foyer 1998) act in scavenging ROS. On the other hand, recently it is broadly accepted that molecules of ROS play a major role in signaling pathways in regulation of plant responses to stress (Suzuki and Mittler 2006; Maruta et al. 2012) and that there are the kindest-specific pathways for signaling by ROS (Mittler et al. 2011). An important source of ROS in plant cells is chloroplasts (Foyer and Shigeoka 2011). Stress conditions limit the processes in the thylakoid membrane, decreasing the supply of $NADP^+$ and favoring the reduction of molecular oxygen (Foyer and Noctor 2009). The ROS generated in the chloroplasts act as a retrograde signal to the nucleus for regulating plant responses to environmental stress (Maruta et al. 2012). The typical response to heat-stress conditions (but also for some other stresses) is production of heat-stress proteins (Kotak et al. 2007). Currently, it is suggested that the production of H_2O_2 is a part of the heat-stress signal transduction pathway leading to the expression of HSPs (Volkov et al. 2006; Konigshofer et al. 2008; Saidi et al. 2011).

Table 12.1 List of papers reporting contribution of particular signaling molecules in heat-stress response

Signal molecule	Species	References
Ethylene	*Agrostis stolonifera* L.	Larkindale and Huang (2004)
	Glycine max L.	Djanaguiraman et al. (2011)
	Arabidopsis thaliana (L.) Heynh.	Larkindale and Knight (2002)
Calcium (Ca^{2+})	*Triticum aestivum* L.	Zhao and Tan (2005) and Tan et al. (2011)
	Festuca arundinacea L.; *Poa pratensis* L.	Jiang and Huang (2001)
	Solanum tuberosum L.	Kleinhenz and Palta (2002)
	Nicotiana tabacum L.	Wei et al. (2009)
Abscisic acid	*Agrostis stolonifera* L.	Larkindale and Huang (2004)
	Zea mays L.	Ristic and Cass (1992) and Gong et al. (1998)
	Arabidopsis thaliana (L.) Heynh.	Baron et al. (2012)
	Malus domestica L.	Brestic et al. (2011)
	Phragmites australis (Cav.) Steud.	Ding et al. (2010)
	Hordeum vulgare L.	Ivanov et al. (1992)
	Cicer arietinum L.	Kumar et al. (2012)
	Oryza sativa L.	Pareek et al. (1998)
Hydrogen peroxide (H_2O_2)	*Glycine max* L.	Ishibashi et al. (2011)
	Phaseolus vulgaris L.	Hüve et al. (2011)
	Sinapis alba L.	Dat et al. (1998)
	Arabidopsis thaliana (L.) Heynh.	Volkov et al. (2006)
	Agrostis stolonifera L.	Larkindale and Huang (2004)
	Cucumis sativa L	Gao et al. (2010) and Liu et al. (2010)
	Capsicum annuum L.	Hu et al. (2010)
Nitric oxide (NO)	*Zea mays* L.	Piterkova et al. (2013)
	Phragmites australis (Cav.) Steud.	Song et al. (2006, 2008)
	Arabidopsis thaliana L.	Gould et al. (2003) and Xuan et al. (2010)
	Oryza sativa L.	Uchida et al. (2002)
Salicylic acid	*Agrostis stolonifera* L.	Larkindale and Huang (2004)
	Vitis vinifera L.	Wang et al. (2010)
	Cucumis sativa L	Shi et al. (2006)
	Vigna radiata L.	Nazar et al. (2011)
	Sinapis alba L.	Dat et al. (1998)
Isoprene	*Phragmites australis* (Cav.) Steud.	Velikova et al. (2004) and Velikova and Loreto (2005)
	Pueraria lobata (Willd.) Ohwi.	Sharkey and Loreto (1993), Singsaas et al. (1997), and Sharkey et al. (2001)
	Phaseolus vulgaris L.	Singsaas et al. (1997) and Sharkey et al. (2001)
	Quercus spp.	Brüggemann and Schnitzler (2002), Delfine et al. (2000), and Copolovici et al. (2005)
	Populus spp.	Calfapietra et al. (2007), Brilli et al. (2007), and Behnke et al. 2007
	Myrtus communis L.	Affek and Yakir (2002, 2003)
	Rhamnus alaternus L.	

Treatment of *Arabidopsis* plants or cells with exogenous H_2O_2 has been shown to induce transcription of heat shock factors (HSF) and small HSP (Volkov et al. 2006; Banti et al. 2010; Saidi et al. 2011). Meinhard et al. (2002) have shown that H_2O_2 has a central position in the transduction of ABA signaling in *Arabidopsis* by regulating the activity of phosphatase. Hence, Maruta et al. 2012 suggested that H_2O_2 is synergistically and antagonistically involved in the signaling pathways for high light response mediated by ABA. Molecules of H_2O_2 from chloroplasts can diffuse outside, leading to signaling processes in the cytoplasm (Mubarakshina et al. 2010). It includes processes like switching activation of the MAP-kinase cascade (Pfannschmidt et al. 2009), which activates nuclear genes in the cell. Among others, the encoding cytoplasmic APX is activated (Vranova et al. 2002; Apel and Hirt 2004; Yabuta et al. 2004; Kreslavski et al. 2012). It is also assumed that H_2O_2 from chloroplast can serve as a redox signal triggering the expression of the gene encoding ascorbate peroxidase in cytoplasm (Karpinski et al. 1999). Moreover, application of H_2O_2 led to an increase of free Ca^{2+} content in cells (Rentel and Knight 2004). The H_2O_2 treatment activated Ca^{2+} channels and elevated Ca^{2+} level in cytoplasm (Pei et al. 2000). Increase in Ca^{2+} leads to activation of the calcium sensor calmodulin triggering the activity of CAT, which downregulates H_2O_2 level. In this respect the H_2O_2 together with a secondary Ca^{2+} signal plays a role in regulation of H_2O_2 homeostasis and thus influences the redox signaling in plant stress response (Yang and Poovaiah 2002; Hung et al. 2005).

12.5.2 Nitric Oxide

Currently, nitric oxide (NO) has been denoted as a key signaling molecule in plants (Neill et al. 2003, 2008). Nitric oxide is a gas soluble in the lipids and water. Durner and Klessig (1999) tagged NO as a gas of ancient origin and ubiquitous importance. NO can be also accumulated in the atmosphere, mainly from industrial pollution. Moreover, plants emit nitric oxide under normal growing conditions (Wildt et al. 1997). Harmful effects of NO on photosynthesis and growth were reported previously, but the effects of NO depend on its concentration. High levels of NO inhibited, whereas low levels enhanced growth of tomato, lettuce, and pea (Anderson and Mansfield 1979; Hufton et al. 1996; Leshem and Haramaty 1996). Takahashi and Yamasaki (2002) have shown that NO can reversibly inhibit ATP synthesis and electron transport in chloroplasts. It was shown that NO is produced in stress conditions where reduction of nitrite by nitrite reductase is declined, which can slow down photosynthesis. Hence, antisense nitrite reductase tobacco plants accumulate more NO and have reduced growth rate (Morot-Gaudry-Talarmain et al. 2002). Another effects of NO on plants were, e.g., hypocotyl and internode elongation in *Arabidopsis*, potato and lettuce (Beligni and Lamattina 2000), increase of chlorophyll content or decrease of chlorophyll loss (Laxalt et al. 1997; Leshem et al. 1997; Beligni and Lamattina 2000), and the senescence delay (Leshem and Haramaty 1996; Leshem 2001). It was shown that NO mediates also the effects of hormones, analogically to hydrogen peroxide (Neill et al. 2002b). At the site of action, hormones induce NO synthesis. Cytokinin induced NO synthesis in tobacco, parsley, and *Arabidopsis* cell cultures (Tun et al. 2001). NO mediates also ABA-induced stomatal closure (Schroeder et al. 2001). Abscisic acid induced rapid synthesis of NO in epidermal cells and guard cells of *Pisum sativum*, *Vicia faba*, and *Arabidopsis* (Desikan et al. 2002; Garcia-Mata and Lamattina 2002; Neill et al. 2002a). NO synthesis was also induced by auxin in the roots of cucumber (Pagnussat et al. 2002). NO is particularly important in signaling within the stress response (Neill et al. 2008). In many signaling cascades involving cGMP, MAPK, CDPK, TFs, H_2S, and other cellular regulators, NO plays a role as a second messenger downstream of hormones (Astier et al. 2010; Saidi et al. 2011; Hancock et al. 2011; Ma et al. 2012; García-Mata and Lamattina 2013; Li et al. 2013). NO probably acts as a Ca^{2+}-mobilizing messenger, and the

mechanisms underlying the cross talk between Ca^{2+} and NO become more clear now (Besson-Bard et al. 2008). Compared to other abiotic and biotic stresses (well reviewed in Arasimowicz and Floryszak-Wieczorek 2007; Neill et al. 2008; Siddiqui et al. 2011; Mazid et al. 2011; Misra et al. 2011), the references on NO signaling in heat stress are less frequent. However, there is strong evidence that NO plays central role in mediation of stress response in high temperature conditions. It was shown that short-term heat stress led to an increase in NO production in alfalfa (Leshem 2001). Endogenous NO levels increased in tobacco and *Arabidopsis* within half an hour after heat shock started (Gould et al. 2003; Xuan et al. 2010). In seedlings of rice under high temperature conditions, Uchida et al. (2002) documented that more green leaf tissues survived and higher PSII quantum yield was recorded in samples treated with low concentration of NO compared to non-treated plants. Neill et al. (2002b) suggest that the protective effect of NO is related to the antioxidative effects. Song et al. (2006) found in callus of reed under heat stress that exogenous application of NO donor alleviated heat-induced ion leakage, tissue growth was less suppressed, and there was higher survival. The increase of the activities of SOD, APX, CAT, and POD was also observed. In callus of stress-sensitive and stress-tolerant ecotypes of *Phragmites australis*, high temperature induced endogenous generation of NO in the tolerant ecotype, while in the sensitive ecotype it was not observed. Treatment with NO increased heat resistance in both ecotypes; however, the enhancement of stress tolerance was higher in the sensitive ecotype. Blockage of endogenous NO production increased necrosis in the tolerant ecotype. These results suggest that NO contributes to protection of tissues against oxidative stress caused by high temperature and that NO plays a role of signal molecule in activation of ROS-scavenging enzymes in heat-stress conditions. Moreover, Song et al. (2008) suggest that thermotolerance mediated by NO is induced by abscisic acid (ABA). Xuan et al. (2010) have shown that NO induces thermotolerance also through heat shock factors (HSFs). In tomato leaves it was shown that NO together with ROS takes part in the regulation of production and accumulation of heat shock protein Hsp70 in stress. Hsp70 accumulation is one part of stress response related to signaling pathways of ROS and reactive nitrogen species. However, the exact mechanism of nitric oxide involvement in the regulation of expression of heat shock proteins and their accumulation in plants under stress needs further investigation (Piterkova et al. 2013).

12.5.3 Cytokinins

Cytokinins are phytohormones regulating plant development but also plastid differentiation and function. The cytokinin biosynthetic pathways are partially located in plastids; it points to the importance of cytokinins in development of chloroplast (Polanska et al. 2007). In the last years, several studies reported the role of cytokinins, plant hormones, in heat tolerance of photosynthesis apparatus. The most recent data show that overexpression of a gene encoding the enzyme adenine isopentenyl transferase (ipt) led to higher levels of cytokinins. It resulted to delay of leaf senescence and increase of stress tolerances, including heat tolerance, in different plant species (Xu et al. 2009; Xing et al. 2009). Chernyad'ev (2009) suggests that the protective cytokinin action on the photosynthetic pathways of plants under stress goes through regulation of structural and functional proteins, including enzymes, that determine many features of the photosynthetic machinery. In this respect, transformation of it in perennial grass species led to changes of protein content in the leaves. The expression level of many proteins involved in different functions, mainly in energetic metabolism, synthesis of proteins and their storage, and the stress defense, was maintained or increased in high temperature conditions (Xu et al. 2010). Similarly, in tobacco plants high temperature (40 °C) led to a transient increase in the level of bioactive cytokinins. Such an increase was associated with a decrease of the cytokinin oxidase/dehydrogenase activity (Dobra et al. 2010).

12.5.4 Isoprene

In the last decades, the role of isoprene and other volatile compounds in high temperature stress resistance is broadly discussed (Sharkey et al. 2008; Monson et al. 2013). It was shown that many plant species emit the volatile compounds, such as isoprene, by leaves (Vickers et al. 2009). The mechanisms of action are not fully understood yet, but it seems to play a role as the signaling molecules in communication among plants in ecosystems. This was documented by the observations that the presence of exogenous isoprene in air leads to an increase of heat resistance (Singsaas et al. 1997), even in some species or transgenic plants, which do not emit the isoprene (Delfine et al. 2000; Sharkey et al. 2001). Emission of isoprene from plants is an important component of biosphere-atmosphere. For plants it is possible to survive without significant emission of isoprene. It is hypothesized that the isoprene is produced only by those plants, in which the benefits from increased thermotolerance outweigh the costs of isoprene producing (Sharkey et al. 2008). The capacity for isoprene emission in *Populus* is given by expression of IspS gene. Sasaki et al. (2005) reported that *Arabidopsis* expressing poplar IspS gene tolerate better heat stress. Similarly, transgenic *Arabidopsis* plants containing an IspS gene originating from *Pueraria* are able to resist the heat-stress level which is lethal for untransformed plants (Sharkey et al. 2005). The poplar trees lacking the IspS expression show more severe damage of photosynthetic apparatus by short heat periods than control trees (Behnke et al. 2007). The effect of isoprene in other abiotic stresses has not been shown. Emission of isoprene is very resistant to drought stress (Tingey et al. 1981; Brilli et al. 2007). Deficit of water that causes nearly total reduction of photosynthetic capacity has only a minor effect on emission of isoprene (Sharkey and Loreto 1993). The mechanism of isoprene in signaling was not explained yet; the only mechanistic explanation of isoprene function is that the isoprene stabilizes lipid membranes (Siwko et al. 2007). It is hypothesized that isoprene protects also against oxidative stress associated with high temperature by decrease of ROS production (Sharkey et al. 2008).

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13 Senescence: Regulation and Signalling

Riyaz Ahmad Dar, Inayatullah Tahir, and Syed Sabhi Ahmad

Abstract

Senescence is a multifaceted, genetically regulated programme, in which cascade of physiological and biochemical changes occur which bring about the deprivation of macromolecules and the recycling of their components to different parts of the plant. Senescence culminates in death of the plant organ as it necessitates cell viability and is often reversible until the late stages of development. The environmental stress factors such as drought, water logging, high or low solar radiation, extreme temperatures, ozone and other air pollutants, and excessive soil salinity, besides inadequate mineral nutrition in soil, negatively influence the senescence. These stress factors disturb the endogenously regulated system of the plant tissue which may result in promoting the process of the senescence. Despite the initiation by environmental factors, the process of senescence is coordinated through a common signalling network by endogenous and exogenous signals involving the signalling molecules ethylene, abscisic acid (ABA), salicylic acid (SA) and jasmonic acid (JA).

Keywords

Senescence • Ethylene • Abscisic acid (ABA) • Salicylic acid (SA) • Jasmonic acid (JA)

13.1 Introduction

Senescence in a broader sense refers to the process of growing old and consists of those changes that are part of genetically programmed events direct to death of a cell which in plants is often referred to as PCD (programmed cell death) (Lerslerwong et al. 2009; Yamada et al. 2009; Shahri and Tahir 2011; Shibuya 2012). Senescence is an unavoidable process that can be seen in particular at the final stage of ontogenesis,

R.A. Dar • I. Tahir (✉) • S.S. Ahmad
Plant Physiology Lab, Department of Botany, University of Kashmir, Srinagar 190006, Jammu and Kashmir, India
e-mail: reyazadar@gmail.com; inayatullahtahir@gmail.com; syedsabhiindrabi@gmail.com

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during which irreversible changes are initiated leading to gradual cell destruction and death of the organism. This process leads to the modification and degradation of the cell components; both at morphological and metabolic level (Fischer 2012). Senescence can be referred to those events that impart endogenous modulation of death or the process that leads to the death of individuals and/or organs. While as PCD can be defined as the programme in which a cell dynamically executes itself and whereby environmental or developmental stimuli trigger explicit cascade of events that terminate in cell death, it also refers to the process by which cells endorse their own death through the instigation of self-destruction systems. In the context of petal senescence, it seems appropriate to use these words almost interchangeably (Rogers 2006; van Doorn and Woltering 2008). Additionally, as senescence generally leads to death, it should be viewed as a programmed cell death (PCD) process or apoptosis (Vicencio et al. 2008). Apoptosis is a Greek term that generally refers to loss of petal or leaf tissues in plant kingdom (Vicencio et al. 2008; Narcin et al. 2005). Despite this, apoptosis is usually confined to animals which refers to morphological transformations such as nucleosome condensation, shrinkage, nuclear blebbing, decreased cell size and above all cell surface changes that lead to their phagocytosis.

13.2 Structural and Biochemical Changes in Senescing Tissues

The senescing cells demonstrate the characteristic biochemical and structural modifications during the process of senescence. In the case of leaf-senescing cells, the notable among structural changes is the disintegration of intracellular organelles such as chloroplasts (Noodén 1988). In chloroplasts, alterations in the structure of grana, its content and lipid droplet establishment are labelled as plastoglobuli. This leads to chloroplasts shrinkage and their conversion to gerontoplasts which is characterised by the crumbling of thylakoid membranes and deposit of the plastoglobulin. Noodén et al. (1997) proposed that chlorophyll breakdown with the concomitant leaf yellowing are used as markers of senescence. After chlorophyll breakdown lipid, protein and nucleic acid dilapidation takes place. Membrane reliability and cellular compartmentalisation are sustained till the last stage of leaf senescence (Pruzinska et al. 2005). A drop in photosynthates during senescence may lead to sugar starvation proceeding to conversion of lipids to sugars. On the contrary, the mitochondrion and nucleus that are essential for the energy formation and gene expression, respectively, remain integral till the completion of senescence (Lim et al. 2007). The premature and hallmark of flower senescence is the membrane relapse which ultimately leads to structural and functional changes in the senescing tissues. The structural change in the petals of senescing *Dianthus* is vesiculation of cytosolic and vacuolar compartments, whereas in the epidermal cells of daylily, the important change was the degradation of vacuolar membrane. The structural changes include the dehydration of senescing tissues, ion seepage, unusual metabolite convey and liberation of cytochrome C from the mitochondrion. The important biochemical changes were the production of reactive oxygen species (ROS); amplification in lipid peroxidation and membrane fluidity; protein hydrolysis followed by hydrolysis of nucleic acids, lipids and sugars; and ultimately reduction in anabolism (Fig. 13.1). Chloroplast deterioration followed by chlorophyll deprivation and the improved shortfall of important proteins such as ribulose bisphosphate carboxylase (Rubisco) and chlorophyll *a/b*-binding protein (CAB). Depending upon the action of several endo- and exopeptidases hydrolysis of proteins to free amino acids (Otegui et al. 2005; Lim et al. 2007). Senescence-related cysteine proteases play an important role in protein deterioration in the vacuole. Hydrolysis and metabolism of the membrane lipids are brought about by lytic acid hydrolase, lipoxygenase, phospholipase D and phosphatidic acid phosphatase in senescing leaves (Thomas et al. 2003). By performing an important part in flower senescence, numerous cysteine proteases have been exposed to be upregulated and further cloned from petals of

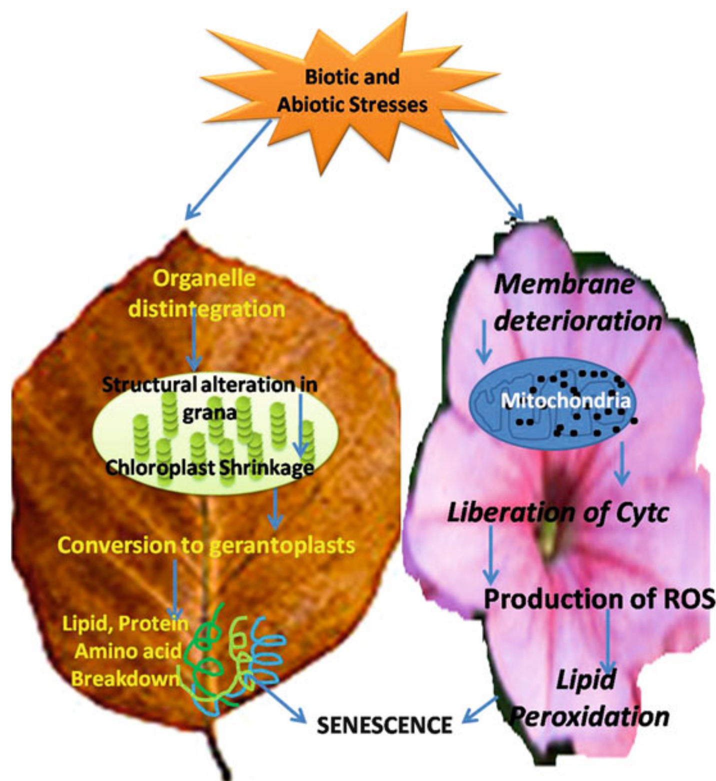

Fig. 13.1 Biochemical changes in senescing tissues

Narcissus, Alstroemeria, Sandersonia and *Petunia*. It has been reported that following the ethylene treatment, most senescence-associated cysteine protease genes increase in abundance. Out of nine cysteine protease genes analysed, six show increased profusion in ethylene-sensitive corollas of *Petunia hybrida* in the course ethylene pursued of petal senescence (Jones et al. 2005). After 3 h of ethylene treatment of *Dianthus caryophyllus*, the expression of cysteine protease *DCCP1* augmented multifold during the petal senescence. After cloning a gene for cysteine protease inhibitor that is expressed profusely in the petals of *Dianthus* during full-opening stage and temporally reduced expression with the progression in flower development that is at senescent stage. It may be concluded that this cysteine protease inhibitor might have an imperative function in the regulation petal senescence by controlling the expression of diverse cysteine proteases. Reactive oxygen species (ROS) generated during the various oxidative reactions is identified to be implicated in natural death of plant tissue together with petals. Hydrogen peroxide is the precursor of these reactive oxygen species; thus, the levels of hydrogen peroxide-regulating enzymes illustrate differential expression during senescence as observed in daylily that augmented activity of superoxide dismutase (SOD) and reduced activity of catalase directs to increased levels of ROS. However, during the senescence of *Dianthus* petals, the increased performance of both ascorbate peroxidase (APX) and catalase, raise in peroxisome number, decrease in the quantity of antioxidants, decrease in membrane transport proteins and redox reactions were reported in several flowers (van Doorn and Woltering 2008).

13.3 Hormonal Regulation of Senescence

The trigger of flower senescence is a very composite phenomenon and administered by the endogenous degree and sensitivity of hormones. The study of plant hormone regulation

on senescence has been reported since a long time back (Tripathi and Tuteja 2007; Fischer 2012). With respect to the trigger of senescence, the role of cytokinins and ethylene has long been implicated and perhaps best understood. But other phytohormones (Schippers et al. 2007; Trobacher 2009) and these phytohormones act as the signalling compound during the process of senescence.

13.3.1 Cytokinins

The role of cytokinins in delaying senescence has long been recognised. The regreening of tobacco leaves was observed after cytokinin treatment, and transgenic plants overexpressing IPT (isopentyl transferase) gene (coding for the enzyme catalysing the rate-limiting step in cytokinin biosynthesis) underneath the SAG_{12} promoter exhibited momentous delay in flower senescence. Based on the experimental findings, the levels of exogenous cytokinins drop during senescence and exogenous application or endogenous enhancement of cytokinin content using the senescence-specific SAG_{12} promoter delaying senescence. The endogenous levels of cytokinins diminish in the leaf-senescing cells, and the genes of cytokinin synthase and adenosine phosphate isopentenyl-transferase (*IPT*) are downregulated and cytokinin oxidase is upregulated during cytokinin synthesis and degradation, respectively (Buchanan-Wollaston et al. 2005).

The strong association between the principal metabolism and anti-senescence effect of cytokinins has been recommended. It has been demonstrated that extracellular invertase is necessary for cytokinin signalling, as cytokinin is no longer able to delay senescence in presence of an invertase inhibitor. Recently, using double-transgenic tomato lines overexpressing both *AtHXK1* (under control of the CaMV 35S promoter) and *IPT* (under control of the SAG_{12} and SAG_{13} promoters), Swartzberg et al. (2011) investigated the interaction of intracellular sugar sensing (by hexokinase) and enhanced cytokinin levels in senescence regulation. Based on visual observations of leaves and on leaf chlorophyll levels, it has been concluded that intracellular sugar sensing by hexokinase is dominant over extracellular sugar sensing (sensing of products of the apoplastic invertase involved in cytokinin signalling), as double-transgenic plants behaved like single-transgenic constitutive *AtHXK1* overexpressors. These experiments also suggest that apoplastic hexoses, in contrast to intracellular sugars, inhibit senescence.

The effect of cytokinins to reduce the onset ethylene biosynthesis and decreased sensitivity to ethylene has been well documented in both petals and leaf tissues. However, similar results were obtained by the exogenous application of kinetin. The interaction between CK and senescence in ethylene-sensitive flowers was gracefully confirmed by Chang et al. (2003), who altered *Petunia* with a SAG12-IPT construct intended to boost CK production at the onset of senescence in leaves. The transformed plants contain more CK content after pollination with the concomitant delay in ethylene synthesis and delay in senescence. The transformed plants were more resistant to exogenously applied ethylene and need longer pulse treatment to induce the endogenous ethylene production and the symptoms of floral senescence. In *Dianthus* petals BA acts as a retarder of the flow of ethylene biosynthesis, involved in natural senescence of *Dianthus caryophyllus*.

13.3.2 Ethylene

The endorsed function of ethylene in senescence has long been confirmed. Ethylene is a gaseous phytohormone formed by plants, and contact of plants to this hormone leads to early senescence in both flowers/petals and leaves (Tripathi and Tuteja 2007). The endogenous levels of ethylene increase during the process of senescence in both senescing leaves and petals with the upregulation of ethylene biosynthetic genes encoding ACC-synthase, ACC-oxidase and nitrilase in many plant species including *Arabidopsis* (Van der Graaff et al. 2006; Lim et al. 2007). Consistent with the notion that the significant delay in leaf senescence in two *Arabidopsis* mutants – ethylene

resistant (etr1) and ethylene insensitive 2 (ein2) – that are scarce in ethylene sensitivity and signal transduction, correspondingly, discloses the importance of endogenous ethylene signalling pathway as a positive regulator of leaf senescence. Delayed senescence was also observed in tomato after the antisense inhibitor of 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase. A MAPK6 is a member of the MAPK pathway that reins plant ethylene signalling during the Arabidopsis senescence regulation. This MAPK is also upregulated by various abiotic and biotic factors and may therefore integrate signals from different pathways influencing plant senescence. Interestingly, the influence of ethylene on senescence is leaf age dependent: Short (1–3 days) ethylene treatments do not induce senescence in young leaves. In older leaves, ethylene effect increases with leaf age, and finally, beyond a certain age, leaf senescence starts even without ethylene as demonstrated by mutant analysis (Buchanan-Wollaston et al. 2005).

Like fruits, ethylene-sensitive flowers also reveal a climacteric rise in ethylene, subsequent pollination and their senescence is triggered by the hormonal changes. In ethylene-sensitive flowers, the senescence-associated changes in petals are observed only after compatible pollination as in the case of *Petunia* which is self-incompatible. The involvement of ethylene in flower development has been studied in great detail in ethylene-sensitive flower systems like *Dianthus, Geranium, Petunia* and orchids. In *Petunia*, *ACO1* is expressed explicitly in senescing corollas and in other floral organs' subsequent ethylene contact, out of four ACC-oxidase (*ACO*) gene members, while *ACO3* and *ACO4* were expressed in developing pistil tissue. The instant and tissue specificity of the amplified expression of *ACO* transcripts concurrent with pollination pursued ethylene production in styles and stigma pursued afterwards by corollas resulting in senescence. Increase in *ACO* transcript in stigma and petals of orchids were allied with pollination. Differential expression of ethylene biosynthetic genes was observed during carnation flower senescence. The genes encoding ethylene receptors show varying expression patterns during flower senescence among species. In carnation, for example, three ethylene-receptor genes, DcERS1, DcERS2 and DcETR1, were identified. DcERS2 and DcETR1 transcripts existed in substantial amounts in petals at the stage of flower development when flowers were fully open. The levels of transcripts for DcERS2 and DcETR1 did not show clear changes in the petals during flower senescence, although the DcERS2 transcript displayed a declining inclination when approaching senescent stage of flower. DcERS1 mRNA was not detected in petals at any stage. Onozaki et al. (2004) reported that ethylene sensitivity is high in the full-opening stage and decreases in the senescing petals of carnation; however, as ethylene receptors are negative regulators of ethylene signalling, it is thought that exhaustion directs to exponential enhancement in sensitiveness.

13.3.3 Abscisic Acid (ABA)

Besides ethylene, ABA is a phytohormone that helps the plant to respond the environmental strains. However, ABA is a key regulator for growth and development of the plant. It has been well documented by several authors (Lim et al. 2007; Tripathi and Tuteja 2007) that the ABA treatment endorses leaf abscission and hastens senescence in certain flowers. After the exogenous application of ABA, unusual early bud abscission and flower senescence was reported in certain cultivars. Being a primary regulator of flower senescence, it also brings about many senescence-associated changes in senescing tissues. These changes comprise ion seepage, enhanced protease activity, lipid peroxidation and expression of novel RNases and DNases. Hunter et al. (2004) proposed that the ABA concentration improved in petal/tepal tissue of senescent flowers as increased content of ABA corresponded with the manifestation of visible signs of senescence in petal/tepal tissue. ABA-mediated senescence initiation in an age-dependent manner was reported quite recently by a membrane-bound receptor kinase (RPK1) rich in leucine content.

This receptor kinase integrates ABA-mediated signals during stress responses, plant growth, stomatal closure and seed germination.

13.3.4 Polyamines (PAs)

PAs play an important role in many biological functions including plant growth and development and during the environmental stresses. Their function in regulation of growth and development in plants has been reviewed by several authors (Kumar et al. 1997; Pandey et al. 2000). There are many forms of PAs, but the most readily available forms of PAs are spermine (Spm), spermidine (Spd) and putrescine (Put) that occur in every plant cell. However, PAs have a well-reputable role in senescence (Tripathi and Tuteja 2007). By acting as anti-senescence agents, PAs have been reported to retard membrane deterioration and chlorophyll loss and increase in protease and RNase activity which untimely helps to retard or slow down the senescence process. By binding to DNA and proteins, PAs stabilise cell membranes. However, they are effective in millimolar concentrations in non-senescent tissues, and their concentration decreases with the age and senescence. Spermine inhibits the senescence in *Dianthus caryophyllus* due to corresponding inhibition of ethylene biosynthesis. This effect of PAs may be due to inhibition of the conversion of SAM to ACC.

13.3.5 Auxins, Gibberellic Acids and Jasmonates

It is very difficult to assign the specific role to auxin in the process of leaf senescence, because of its participation in most of plant developmental phases. However, the endogenous levels of auxin increase during the senescence which suggests its implication in the process of senescence (Sexton and Roberts). During age-dependent leaf senescence, the IAA biosynthetic genes encoding IAA1d oxidase (AO1), tryptophan synthase (TSA1) and nitrilases (NIT1-3) are upregulated (Van der Graaff et al. 2006).

The role of auxins and gibberellic acids in flower senescence has not been well established. However, the exogenous application of auxins in some ethylene-sensitive flowers enhances their senescence. Jones and Woodson (1999) convey that 2,4-D, a synthetic auxin, persuade the expression of ACC synthetase genes in the styles, ovaries and petals. A transitory raise has been reported in the mRNA of an Aux/IAA gene following the application of auxins in the petals of carnation (Hoeberichts et al. 2007). Acting as an antagonist to ethylene, GA application delays the onset of senescence in carnation cut flowers with reduced ethylene production.

Jasmonate has a well-characterised role in the process of senescence and regulates the senescence in isolated oat (*Avena sativa*) leaves. Methyl jasmonates (MeJA) are negative regulators of senescence as exogenous application of MeJA to isolated *Arabidopsis* leaves escorts to a hasty loss of chlorophyll content and decreased photochemical efficiency of photosystem II (PSII) and augmented expression of *SAG*s such as *SEN4, SEN5* and γ *VPE*. The observation that JA-dependent senescence is faulty in the JA-insensitive mutant *coronatine-insensitive 1* (*coi1*). This study provides a realistic approach for the role of JA signalling pathway in sustaining leaf senescence. Jasmonates have an inspiring consequence on flower senescence. Jasmonic acid besides numerous other metabolites rouses the ethylene production by enriching the levels of ACC and promotes the flower senescence of orchid species. However, after a 50h pollination provoked senescence, neither lipoxygenase activity nor jasmonic acid content changed in orchid petals.

13.4 Nonhormonal Regulation of Senescence

Leaf senescence is a dynamic process that can be activated by a multitude of internal and external cues. It diminishes photosynthetic carbon fixation but is important for nutrient recycling particularly nitrogen (Uauy et al. 2006). Accumulation of hexose in the ageing

leaves acts a signal for acceleration or initiation of senescence in annuals (Masclaux-Daubresse et al. 2005; Parrott et al. 2005, 2007; Pourtau et al. 2006; Wingler and Roitsch 2008; Wingler et al. 2009). The role of sugars in leaf senescence was elucidated by various workers. Some researchers believed that sugars act as signal molecules for regulation of senescence, but van Doorn was of the opinion that sugars might not always be a direct cause of senescence but may act via a number of other signals (Wingler and Roitsch 2008; Wingler et al. 2009). Exogenous application of sucrose promotes opening of cut flowers, and delayed senescence, and having no influence on the abscission of petals (Arrom and Munne-Bosch 2012), however changing the hormonal balance in floral tissues. A reasonable good amount of sugar is required for floral bud opening, as substrates for respiration and maintaining osmotic balance of cells. As cut flowers are devoid of any sugar source, the exogenous supply of sugars such as sucrose, glucose and trehalose is essential in promoting their opening.

The stress-generated factors and reactive oxygen species (ROS) containing unpaired electrons including singlet and triplet oxygen, superoxide, nitric oxide and hydroxyl radicals affect cells either through damage (by reacting with DNA, proteins, lipids, etc.) or by acting as signalling molecules, e.g. in hormonal signalling or in response to abiotic and biotic factors (Foyer and Noctor 2005; Pitzschke et al. 2006; Møller et al. 2007; Kazemi and Ameri 2012). Of particular importance to this, the number of evidences indicate that ROS contribute in senescence initiation and signalling (Zimmermann et al. 2006).

SA is a phenol that acts as a plant growth regulator, regulating a number of plant physiological processes including photosynthesis (Sawada et al. 2006; Munne-Bosch 2007). SA is an impending nonenzymatic antioxidant that increases stress tolerance during abiotic and biotic stresses. Sawada et al. (2006) and Kazemi et al. (2011b) showed that SA can prevent ACC-oxidase activity (Ansari and Misra 2007; Mba et al. 2007; Mahdavian et al. 2007; Canakci 2008; Kazemi et al. 2012); also SA appears to act as a germicide that decreases the concentration of bacterial species which occlude the xylem vessels and block them and obstruct the normal flux of water via the stem in cut *Dianthus* flowers. Increased antioxidant activity of enzymes delays the onset of hydrolysis of structural components of cells thereby diminishing the production ROS and a concomitant decrease in ACC oxidase sensitivity and activity. A role for salicylic acid in the regulation of developmental senescence was first demonstrated by Morris et al. (2000) using mutant's defective in SA signalling (*npr1* and *pad4*) as well as *NahG* transgenic plants. In these experiments, induction of the *SAG12* protease gene was dependent on the presence of SA, and mutants defective in SA signalling showed delayed yellowing.

Most of the genes encoding hydrolytic enzymes get expressed during the senescence and bring about the structural disassembly of cell components including macromolecules. Further, genes encoding nucleases, stress responsive enzymes, nitrogen mobilising enzymes and carbohydrates including many transcription factors showed increased expressions during the senescing leaves.

13.5 Reactive Oxygen Species and Nitrogen Species (ROS and RNS) in Senescence Signalling

Molecules having oxygen with unpaired electrons (reactive oxygen species) and nitrogen species (NO) play a critical role by acting as signalling molecules, for example, in hormonal signalling or in biotic and abiotic stresses. Among the ROS species, stress has been laid on H_2O_2 as it is relatively small, uncharged with longer half-life (~1ms) and thus can easily pass the membranes. Because of these reasons, the central position has been attributed to this molecule in signalling pathways. However, nitric oxide (NO) has been revealed to be implicated in several H_2O_2-mediated pathways either an antagonistic or synergistic mode of action. During the ABA-intervened drought- induced leaf senescence,

the ROS complex upstream regulator has been identified. The ROS production by the drought-responsive NAC transcription factor AtNTL4 (ANAC053) directly enhances the activity of gene promoters coding for enzymes involved in ROS biosynthesis (Lee et al. 2011). It has been proposed that the ABA-H_2O2-NO signalling cascade induces stomatal closure. The generation of NO by H_2O_2 induction has been reported in several plants like Mung bean, Arabidopsis and other plant species (Lum et al. 2002; He et al. 2005; Bright et al. 2006). The link between the NO generation and H_2O_2 can be analysed by the exclusion of H_2O_2 in addition to jamming of calcium channels (Neill et al. 2008). The cytoplasmic H_2O_2 can also unswervingly trigger explicitly Arabidopsis MAP triple kinase, AtANP1, which instigate a phosphorylation cascade concerning two stress AtMAPKs, AtMPK3 and AtMPK6. During this discourse MPK6 phosphorylates and thus trigger NR2 ensuing in improved NO assembly (Wang et al. 2010). An additional point of crosstalk among the NO and H_2O_2 signalling pathways has been revealed by positional cloning of rice gene (*NOE1*) coding for catalase, the elimination of which escorts to augmented H_2O_2 contents which in turn increases the activity of NR and leads to eminent NO concentrations. The exclusion of excess NO rearranges the cell death symptoms of the *noe1* mutants revealing an obliging function of H_2O_2 and NO during induction of PCD. Here, exclusively S-nitrosylated proteins were recognised, and overexpression of a rice S-nitrosoglutathione reductase could also raise the cell death signs (Aihong et al. 2012).

13.6 Conclusions

Senescence is not a simple wear and tear mechanism, but is a multifaceted, genetically regulated programme, in which series of biochemical and physiological changes occur which brings about the degradation of macromolecules and the recycling of their components to different parts of the plant. The changes are brought about by plant hormones. Nowadays senescence is a talk of the town as per flower horticulture is concerned. Tremendous work is being carried out with regard to the flower senescence. This review tries to bring the latest information regarding the senescence regulation, mechanism and signalling. We also tried to elucidate the role of growth regulators, reactive oxygen species and nitrogen species in senescence regulation and signalling.

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14 Molecular Builders of Cell Walls of Lignocellulosic Feedstock: A Source for Biofuels

Neeru Gandotra

Abstract

Biofuels are an alternative source of renewable and sustainable energy which can be obtained from lignocellulosic feedstock (crop or non-crop energy plants), algae, or as a by-product from the industrial processing of agricultural/food products, or from the recovery and reprocessing of products such as cooking and vegetable oil. Such biofuels are generally in the form of either bioethanol or biodiesel or biobutanol. It is necessary that improvements be made at every stage during the processing of biofuel starting from enhancing the ability of the plant to maximally utilize the solar energy to fix the CO_2 into biomass and generate greater amounts of cellulosic material. The next step in the process would be to separate the cellulose from the lignin in a cost-effective way. And finally extract ethanol from this cellulose using various methodologies such as fermentation and/or cellulose pyrolysis. Engineering the steps involved in releasing the cellulose from the other cell wall components especially lignin would reduce the cost of generating biofuels from lignocellulosic materials. Hence, an in-depth understanding of the molecular components that are involved in either the regulation or biosynthesis of lignin and consequences/limitations of altering those pathways and redirecting the flux to alternate pathways are discussed.

Keywords

Lignin • Biofuel • Lignocellulosic feedstock • Molecular builders

14.1 Introduction

Biofuels generated in a sustainable and environmentally friendly way is being increasingly considered as an alternative source of energy over the traditional fossil fuels due to their adverse effect on the environment and limited supply and reserves of fossil fuel. It is projected that there

N. Gandotra (✉)
Department of Molecular, Cellular and Developmental Biology, Yale University, New Haven, CT, USA
e-mail: neeru.gandotra@gmail.com

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would be an increase in the world energy consumption by 54 % between 2001 and 2025. Increasing demands accompanied by the low net greenhouse gas emissions have put forth the usage of lignocellulosic feedstocks to produce liquid biofuels.

Although the idea of using plants to generate energy has been around for a couple of decades, its use to replace the fossil fuels has only recently been recognized. The United States and Brazil account for almost 90 % of the global production of bioethanol, while Europe produces 53 % of biodiesel. Brazil has replaced petroleum-derived oil for their transportation needs with ethanol produced from sugarcane (Chaddad 2010). There has been an ever-increasing effort in the USA and around the world to replace the existing fuel sources with biofuels. About 30 % of fuel consumption is to be replaced by biofuels as directed by the US government.

14.2 Lignocellulosic Feedstocks as Candidates for Biofuels

So far grasses mainly of the C4 type have been a major contributor to the lignocellulosic biofuels. C4 grasses demonstrate a higher efficiency in photosynthesis by compartmentalizing the process of photosynthesis between the bundle sheath and mesophyll cells and thereby increasing the carboxylation activity of the RuBisCO enzyme to fix CO_2 and reducing the loss of carbon/energy via photorespiration. By doing so, they gain an advantage in their nutrient and water use efficiencies. All of this makes them favorable candidates with regard to biomass production. Additionally, the cell walls of grasses are special with regard to their cell wall content. Grasses contain type II cell walls, while dicots contain type I cell walls. The main polymer components of cell walls of grasses are cellulose, arabinoxylans, and lignins. Grass cell walls are characterized by a high proportion of hydroxycinnamic acids, i.e., ferulic acid (FA) and p-coumaric acid (pCA). Lignin and phenolics bound to the cell walls hinder the release of cellulose for conversion to bioethanol. The biofuel crops such as sugarcane and maize are the first-generation feedstocks. More recently the second-generation feedstocks such as switchgrass and Miscanthus are gaining importance as biofuel feedstocks since they do not compete with hampering the food security.

14.3 Challenges Associated with Incorporating Biofuel from Lignocellulosic Plants to Existing Setup

(a) One of the challenges is the inefficiency in procuring cellulose from the lignocellulosic plants due to the presence of lignin. Removal of lignin tends to be costly.
(b) The quality of the fuel obtained is different from that obtained using fossil fuels in terms of their number of carbon atoms.
(c) There is a need for specialized equipments to incorporate the bioethanol into the existing setup.
(d) Ethanol is difficult to transport and it needs to be mixed with the conventional fuel at the delivery point.

In order to overcome some of the challenges mentioned above, we will discuss the biosynthesis and regulation of lignin to understand and determine the steps that can be engineered in planta to modify and/or reduce lignin content thereby improving the recalcitrance of lignin with cellulose.

14.4 Lignin Biosynthesis

Lignin is a phenolic polymer that is mainly derived from the hydroxycinnamoyl alcohols. It is found mostly in tracheophytes. Lignin provides structural rigidity for land plants to stand upright and strengthens the cell wall of tracheary elements that conduct water from the roots all the way to the tip of the plant and withstand the negative pressure from transpiration. Lignin deposition in the primary wall starts after the secondary wall formation, and the factors and mechanism that control the deposition of lignin in both types of cell wall appear to be under the

control of the same genes/enzymes (Harrington et al. 2012). There are three types of lignin, H (*p-hydroxyphenyl*), G (guaiacyl), and S (syringyl), that eventually make the plant cell wall in most grasses. The ratios and types of lignin depend on the species type, stage of development, and cell type. Grass cell wall is made up of all three types with S and G being the predominant types. A feature of grass cell wall is that it is made up of relatively high amounts of the H type of lignin compared to dicots that contain trace amounts of it (Barrierre et al. 2007; Dixon et al. 2001). Another unique feature of grasses is that they contain relatively high amounts of p-hydroxycinnamic acids, particularly pCA and FA (ferulic acid). Earlier studies have shown that these acids play an important functional role in the incorporation of lignin into the cell wall (Grabber et al. 2004). Due to the impact of the amounts of lignin on the initial steps of lignin polymerization, it has been hypothesized that FA might play a role in determining the sites of nucleation for lignification. Phylogenetic analysis done by Xu et al. (2009) suggests that expansion of the lignin biosynthetic gene families occurred after the speciation of mono- and dicotyledons which explains so many differences between monocot and dicot cell wall.

The lignin biosynthetic pathway essentially consists of ten steps (Fig. 14.1). A detailed description of the phenotypes of the mutant and transgenic plants that are affected in the lignin pathway is described in the review by Harrington et al. (2012). In general, mutation in the lignin biosynthetic genes results in reduced lignin content along with a change in the ratio of the H, G, and S content that ultimately results in a higher enzymatic digestibility of these mutants compared to wild type. If the mutation results in a visible phenotype, it resembles the brown midrib mutant phenotype. The mutations so far described have been identified in the 4CL, CCR, COMT, and CAD genes in the lignin biosynthetic pathway. Future research needs to be done to characterize the role of C4H, HCT, CCoAOMT, and F5H in terms of plant growth and lignin content.

14.5 Lignin Regulation

Natural variation for cell degradability appears to be at the regulatory mechanisms rather than the biosynthetic pathway based on the co-localization of QTLs involved with the lignin or cell wall degradability and genes present at those physical locations (Harrington et al. 2012).

Plant cell walls are highly complex and dynamic in nature. The composition of cell wall not only differs among different cell types but also varies in different microdomains of the same cell. This is achieved in part by the regulatory mechanisms controlling biosynthesis, targeted secretion, and assembly of wall components that provide such heterogeneity within and among the cell types. A number of factors such as hormones, cytoskeletal components, glycosylphosphatidylinositol-anchored proteins, phosphoinositides, sugar nucleotide supply, and coordination of wall biosynthesis are implicated in the process of cell wall biosynthesis and deposition (Zhong and Ye 2007).

There are specific transcription factors that regulate the secondary wall biosynthesis in each cell type, and the cell wall patterns appear to be initiated by the microtubule organization in those cell types. However, some NAC transcription factors might play a role in secondary wall deposition patterns although those mechanisms are not so clear yet. Transcription factors in the NAC and MYB family are the key master regulators of the secondary wall biosynthesis. These master regulators are active in different cell types, but their downstream targets appear to be the same set of genes involved in the biosynthesis of secondary wall components such as cellulose, lignin, and xylan (Yang et al. 2013). For instance, the differentiation of the vessel and fiber cell starts independently of each other, and the fiber secondary cell wall is under the control of NST1 and NST3/SND1 master regulator, whereas the vessel is under the control of VND6 and VND7 but thereafter shares the same regulatory network for lignin, cellulose, and xylan biosynthesis (Wang and Dixon 2012). These secondary wall-related NAC transcription factors (SWNs)

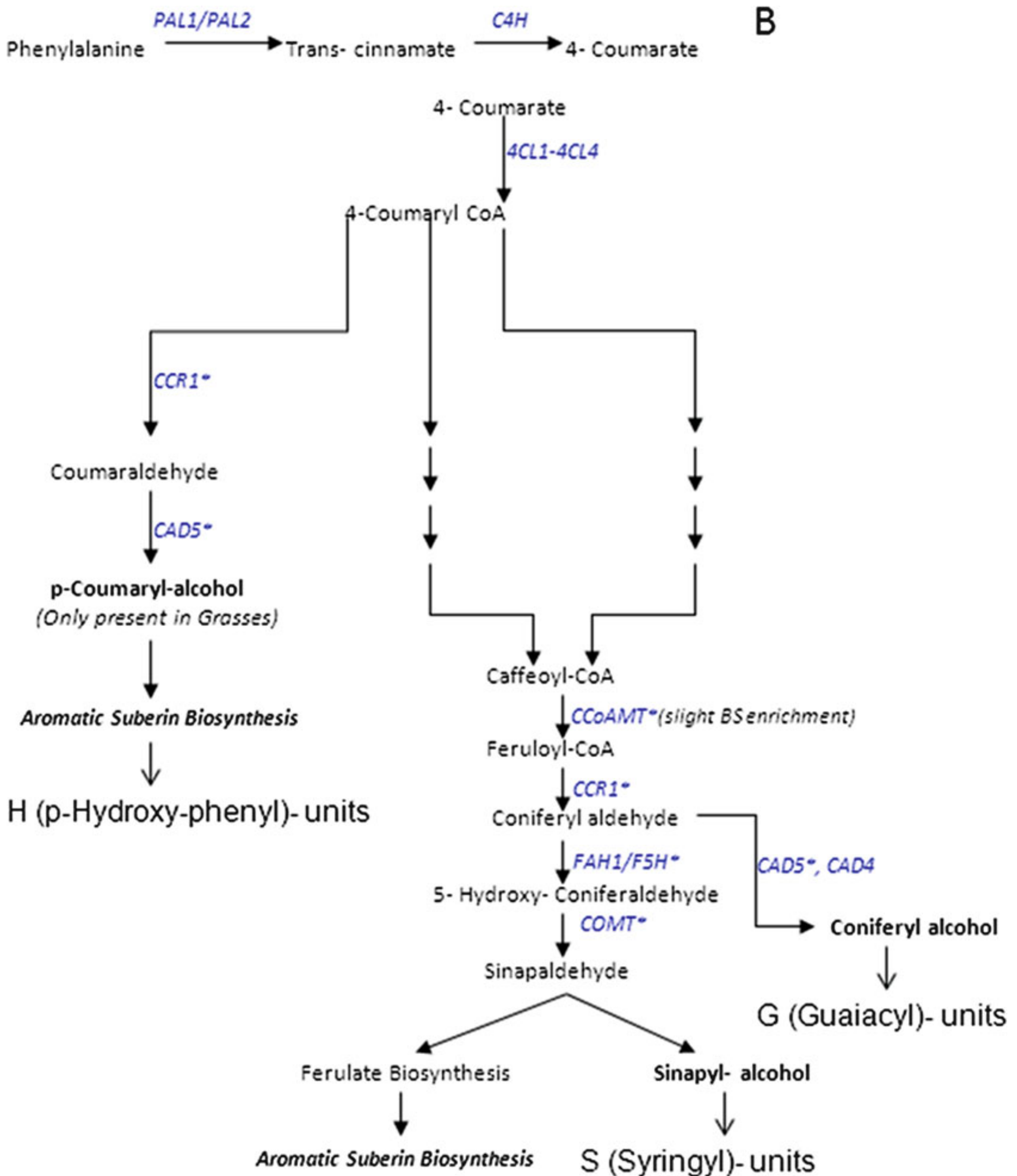

Fig. 14.1 Biosynthetic pathway of Lignin

function as the first layer of master regulators, and the MYBs act as the second layer of master switches in secondary wall formation (Fig. 14.2). The SWNs are under the control of both positive and negative feedback regulation. It was shown in pith cells that AtWRKY tx factor functions as a negative regulator of SWNs (NST2) to maintain parenchymatous identity of these cells (Wang et al. 2010).

The biosynthetic pathway genes for formation of the *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) monolignol building blocks contain AC cis-elements in their promoter regions. Both the positive and negative MYB transcription

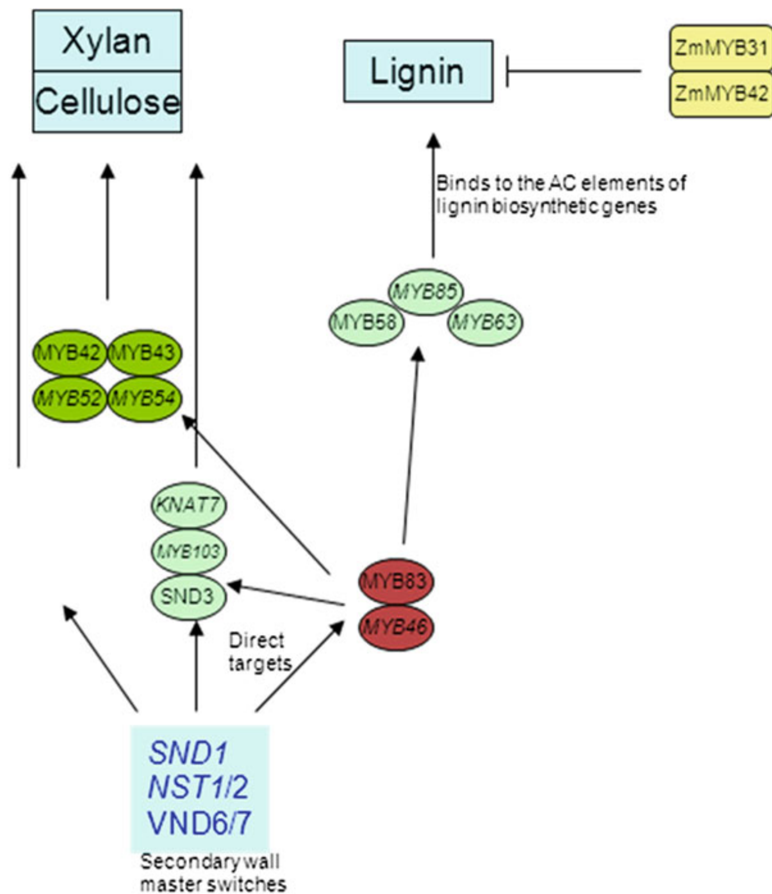

Fig. 14.2 Regulation of secondary cell wall biosynthesis

factors have been reported to bind to the AC cis-element and regulate the lignin biosynthesis. It is still unclear if they bind to the same site and how lignin synthesis is coordinated. The AC element has been proposed to coordinate G lignin biosynthesis (Zhao and Dixon 2011). However, genes specific for the synthesis of S lignin such as F5H are directly activated by NST1 (Zhao et al. 2010). Further work is required to shed some light into the details of how these regulators control lignin composition and content.

14.6 Areas for Improvement

It has been suggested that lignin deposition is a dynamic and adaptive process (Vincent et al. 2005). Lignin deposition and biosynthesis vary in terms of cell type, stage of development, tissue type, specificity, and plant species. Interfering with one of the steps in the lignin biosynthetic pathway at the whole plant level could have several detrimental effects such as loss of mechanical strength, reduced vessel integrity leading to reduced water and nutrient transport, and reduced/altered accumulation of the different subunits in the cell wall that could adversely affect the plant's response to abiotic stresses due to changes in the composition of the stress/defense lignins. Hence, a targeted approach is needed. A recent work by Yang et al. (2013) shows such an example where they document the use of cell-specific promoters fused to secondary cell wall deposition genes. Directing the synthesis of lignin just to the vessels and creating an artificial feedback loop to enhance the expression

of the NST1 (master regulator of secondary cell wall biosynthesis in fiber cells) gene in the fiber cells to generate enhanced polysaccharide deposition in its cell wall without lignin resulted in heathier plants with increased amounts of sugar that released easily from cell walls of these plants.

14.7 Effects of Modification

Lignin is an important part of the cell wall polymer that is required not just to provide mechanical strength but also to act as a physical barrier for protection from the invasion of pathogens. It is also important for growth and development. It is important that deep loss of lignin will render the plant unhealthy and affect its ability to grow and develop and to tolerate any kind of abiotic/biotic stress. However, moderate changes in the lignin content and changes in the composition have improved the release of cellulose from the cell wall after pretreatment. Here, we look at the effect of altered lignin mutants in terms of their ability to fight diseases.

14.8 Resistance to Diseases

Lignin synthesis has long been correlated with the plant's defense mechanism against pathogens especially since lignin is synthesized at sites of infection. Lignin acts as a physical barrier to invasion and growth of the pathogens. Could modifying the lignin structure have an effect on the plant's resistance to diseases? The phenylpropanoid pathway that synthesizes monolignols is further involved in the synthesis of other phenolic compounds such as phenolic phytoalexins, stilbenes, coumarins, and flavonoids. Some of these have roles in plant defense. An important plant defense hormone salicylic acid is derived from this phenylpropanoid pathway in some plants. Certain abiotic stresses also induce the expression and activity of the enzymes involved in this process. Altering or reducing the lignin biosynthesis could therefore have serious outcomes in the ability of the plant to withstand stress. However, some of the published reports suggest that lignin modification may not lessen the disease resistance to pathogens.

Most of the published work on plants with modified lignin via interfering with the lignin biosynthetic genes and plant-pathogen interactions is done on plants that are not bioenergy feedstock such as Arabidopsis and tobacco. This work suggests that knocking down the function of PAL, the first committed step to monolignol biosynthesis results in reduced susceptibility of tobacco plants to *Cercospora* spp. the causal agent of frogeye disease. Overexpressing PAL in tobacco resulted in an increase in the SA and other defense-related compound chlorogenic acid. Increased SA levels provided the plants resistance to the *Cercospora nicotianae,* but the resistance to TMV was unchanged. These plants however showed increased susceptibility to the insect *Heliothis virescens*. Similarly T-DNA insertion in all four PAL genes resulted in increased susceptibility to the bacterial pathogen *Pseudomonas syringae* (Huang et al. 2010). Since PAL is involved in the synthesis of a whole range of phenolic compounds, the changes in the resistance to pathogens cannot be directly attributed to changes in lignin.

Interestingly, however, reducing the expression of the HCT gene in Arabidopsis and alfalfa via antisense/RNAi results in the activation of the defense responses. In both of these genera, antisense/RNAi suppression of the HCT gene resulted in reduced growth and lignin content. It was noteworthy that even in the absence of the disease, the HCT-knockdown plants had elevated SA levels compared to the WT plants (Gallego-Giraldo et al. 2011a, b) in alfalfa. Reducing the expression of a gene SID2 (SA induction deficient 2–2) that is part of the isochorismate pathway leading to the synthesis of SA helped recover some of the growth phenotype defects. It is however unclear if the increased levels of SA can be attributed to re-funneling of some of the compounds of the phenylpropanoid pathway. There was a significant accumulation of pectin-related compounds in the cell wall of these plants which could provide additional defense to these plants.

Similar results of either increase in pathogen resistance or no change were obtained when

COMT expression was knocked down via antisense/RNAi suppression in Arabidopsis and tobacco (Sattler and Funnell-Harris 2013; Quentin et al. 2009). However, suppressing the CAD gene function resulted in increasing the susceptibility of the plants to a range of pathogens (Sattler and Funnell-Harris 2013). It is important to note that CAD suppression is an important target of the bioenergy feedstock to reduce lignin content. Similar results of increased susceptibility were reported for mutation in the gene F5H in monolignol biosynthetic pathway in Arabidopsis (Huang et al. 2009).

Although the work done on the plant-pathogen interaction in the modified bioenergy feedstocks is minimal, more light is shed from the brown midrib mutants (bmr) of maize and sorghum that have long been shown to contain reduced lignin contents. There are 5 bmr loci in maize and 7 in sorghum (Sattler and Funnell-Harris 2013).

bmr loci of maize and sorghum

	Homologous gene
Sorghum	
bmr2	4CL
bmr6	CAD
bmr12	COMT
Maize	
bmr3	COMT
bmr1	CAD

Interestingly, studies done under field conditions on the bmr mutants have revealed that mutations in the phenylpropanoid/lignin biosynthetic pathway either provide these plants with increased resistance to pathogens or cause no change in resistance (Sattler and Funnell-Harris 2013). The lesion lengths in bmr6, 12 were considerably smaller or same when inoculated with *Fusarium thapsinum* compared to their wild-type relatives and across different genetic backgrounds (Sattler and Funnell-Harris 2013; Funnell and Pedersen 2006; Funnell-Harris et al. 2010). However, the fungal growth was greater in the bmr12 plants in healthy-appearing tissues outside the necrotic discolored tissue that is defined as the lesion. Inoculations with another fungal stock pathogen *Macrophomina phaseolina* that causes charcoal rot showed that brown midrib mutants were not more susceptible to this pathogen. However, these studies relied on artificially inoculating the fungi and do not take into consideration the stalk strength that may affect the rind penetration resistance. In general mutations in the bmr genes resulted in affecting at least three different steps, i.e., 4CL, COMT, and CAD, and all of them seemed to not make these plants any more susceptible to stalk rot pathogens but may even cause an increased generalized resistance to pathogens. There could be several reasons for this: (1) There is evidence that they are hampered in their ability to synthesize structural lignins, but research needs to be done to evaluate changes in the synthesis of "defense lignin/stress lignin" in response to pathogen attack. (2) Blocking a step in the monolignol biosynthesis would result in the increase in the accumulation of the precursors that could be directed to the synthesis of other compounds that would have roles in defense response. (3) Perturbations in the synthesis of lignin a component of the cell wall might result in a generalized cell wall response that might provide additional defense to the plant.

In general reducing lignin content and altering its composition does not seem to have a tremendous change in the susceptibility of these bioenergy feedstocks to pathogens, but a case-by-case approach including field trials would need to be evaluated to determine pathogen susceptibility.

14.9 Future Directions/Emerging Technologies

Genetic modification of plants to alter lignin content can improve lignin degradation. A list of newly discovered lignin monomers has shown that lignin is able to readily copolymerize alternative units that are produced by incomplete synthesis of monolignols. An example of this has been shown in a biomimetic system by polymerizing coniferyl ferulate together with normal monolignols into the primary cell walls of maize (Grabber et al. 2008). The modified lignin incorporates easily breakable ester bonds within its backbone and

hence is easily degraded at lower temperature and under alkaline conditions. This is an area that also requires a huge study at the systems level to identify the consequences of such change on the regulation of the pathway itself and a whole range of related pathways that may alter the ability of the plant to respond to stress and pathogens. It is eminent that such studies be evaluated in the field trials where the plants are exposed to various conditions and stresses caused by environment and pests that are sometimes not possible to mimic under laboratory or greenhouse conditions. Besides the clear benefits of biotechnology in the generation of such GM plants, it is important to harness a whole host of natural mutants that may have modified cell walls that easily release the sugars from the plant cell wall.

The US Department of Energy has several recommendations to emerging alternative fuels, and those relevant to the context will be discussed further. Drop-in biofuels that are under research and development phase are substitutes for existing diesel, gasoline, and jet fuel which typically fuel vehicles that are not good candidates for electrification. These drop-in biofuels are expected to drop in directly into the existing infrastructure without any compatibility issues which are a barrier to ethanol and biodiesel.

There is more than one way to produce such fuel, and some of the potential technological pathways include upgrading alcohols to hydrocarbons by converting sugars to hydrocarbons either catalytically or via fermentation. Another would be to process biomass into bio-oil via pyrolysis or liquefaction.

There are some clear benefits of these drop-in fuels especially since they are expected to be substantially similar to their petroleum counterparts and hence do not require major modification to the existing infrastructure. They contribute to fewer greenhouse gas emissions and offer greater flexibility by allowing for replacement of diesel, jet fuel, and gasoline for products from various feedstock and production technologies.

There is a need to generate plants that are optimized for the production of biofuel via genetically modifying their cell walls without compromising their biomass or toleration to diseases and stress.

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Recent Trends in Jasmonate Signaling Pathway

15

Tariq Ahmad Dar, Moinuddin, Nadeem Hashmi, Mohd Idrees, and Akbar Ali

Abstract

Jasmonates (JA) are a new class of plant hormones that has recently been worked out regulating the interweaving plant responses against invading microbes, herbivores, mechanical damage, and other environmental stresses. Their interactions are extensive working at the forefront of root or shoot tissues through volatile regulators, or they are regulated interdependently through the cross talk of other established growth regulators. Jasmonates, the product of octadecanoid pathway, have recently emerged as a versatile regulator of plant physiological and defense responses after salicylates. Most of the components of their signaling pathway have been identified through mutant studies. A progress towards the interaction of JA with other phytohormones is underway. Present review sheds the light on the recent progress made in identification and working mechanism of jasmonate receptor complex, its downstream signaling pathway, and the molecular interaction of its components with the signaling pathways of some other plant hormones.

Keywords

Jasmonates • Receptor complex • Cross talk • Phytohormones

15.1 Introduction

Plants respond by changing their growth and developmental processes after receiving the light, chemical, and various other environmental signals. A multifarious network of interactions is involved in signaling pathways to coordinate the physiological responses such as flowering, fruit ripening, germination, photosynthetic regulation, nutrient absorption, and shoot or root development. Plants being sessile organisms have no choice other than to adapt their growth and form

T.A. Dar (✉) • Moinuddin • N. Hashmi
M. Idrees • A. Ali
Plant Physiology Section, Department of Botany, Aligarh Muslim University, Aligarh 202 002, India
e-mail: dartariq21@gmail.com; moinuddin.bt@gmail.com; nadim.husain@yahoo.com; idreesamu@gmail.com; akbar.agra.alig@gmail.com

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to the changing environment and need to coordinate their growth and physiology accordingly. Plants need appropriate integration of the different environmental factors with their developmental processes in order to survive, and certainly the central role played in this integration is that of phytohormones. In addition to the traditional phytohormones like auxin, cytokinin, gibberellin, abscisic acid, and ethylene (Santner and Estelle 2009), several new compounds are also recognized as plant hormones including jasmonates, salicylates, nitric oxide, strigolactones, and brassinosteroids (Grun et al. 2006; Vert et al. 2005; Browse 2005; Loake and Grant 2007; Gomez-Roldan et al. 2008; Umehara et al. 2008; Santner and Estelle 2009). In some cases plants also control growth by using peptides as hormones (Jun et al. 2008). Plant signal transduction shows extensive cross talk among different pathways unlike the linear chain of events as was previously thought. Signaling cascades, being complex in nature, are coordinated and integrated by different positively and negatively acting signaling molecules; hence, the transduction pathways need to be highly specific and sensitive. Interaction of signaling pathways controls the excess input of signals received by the plants from different signaling cascades; this enables the plants to execute the appropriate physiological and developmental responses.

Jasmonates are members of the oxylipin family (oxygenated derivatives of fatty acids) synthesized through the oxidation of unsaturated fatty acids. JA, synthesized in plants via octadecanoid pathway, are similar to animals' anti-inflammatory prostaglandins in structure and biogenesis (Creelman and Rao 2002; Wasternack and Hause 2002), are considered as plant growth regulators (PGRs), and act as the intracellular signal compounds in the elicitation of plant defense responses (Creelman and Mullet 1997). Methyl jasmonate (MeJA) is an effective elicitor for stimulating secondary metabolite production in plant tissue culture studies (Dicosmo and Misawa 1995). Exogenous application of MeJA has been shown to potently elicit the secondary metabolite biosynthesis of various plants (Gundlach et al. 1992; Pauwels et al. 2009). In various plant systems the role of JA has been confirmed in simulating the gene expression (Menke et al. 1999; van der Fits and Memelink 2000), phytoalexin accumulation, and other antimicrobial and antioxidative secondary metabolites (Gundlach et al. 1992; Aerts et al. 1994; Gantet et al. 1998; Lee-Parsons et al. 2004). Various workers have, in fact, reported the JA-regulated diverse plant defense responses in different plants (Kunkel and Brooks 2002; Wasternack and Hause 2002). Direct plant defense is mediated by the JA-regulated phytochemicals like proteinase inhibitors (PIs) and polyphenol oxidases (PPOs) which are known to check the growth, feeding, and reproduction of the invader. JA also help in orchestrating "indirect" responses. A classical example of the indirect defense-mediated response by JA is the production of extrafloral nectaries, which attract the insects beneficial to plants, with the corresponding reduction in the numbers of herbivores as well as the damage caused by the herbivores (Arimura et al. 2005; Heil 2004; Heil et al. 2001; Linsenmair et al. 2001). JA mediate plant-to-plant or intersystemic signaling (Farmer 2001) similar to salicylates (Hayat et al. 2012) which is demonstrated in sagebrush (*Artemisia tridentata*) that MeJA induces proteinase inhibitors in the surrounding plants (Farmer and Ryan 1990). Jasmonates provide resistance against insects and diseases, which is supported by several lines of evidences. First, JA get accumulated in wounded plants (Creelman et al. 1992) and in plant or cell cultures which are treated with elicitors of pathogen defense responses (Gundlach et al. 1992). Second, JA activate genes for PIs that protect plants from insect damage (Johnson et al. 1989). Jasmonates are also found to activate expression of genes encoding antifungal proteins such as osmotin (Xu et al. 1994), thionin (Becker and Apel 1992), and the ribosome-inactivating protein RIP60 (Chaudhry et al. 1994). Jasmonates modulate the expression of cell wall proteins such as pathogenesis-related proteins (PRPs) which provide immunity to the infections (Creelman et al. 1992). Further, JA induce genes involved in the biosynthesis of phytoalexin (*Chs, Pal*, HMGR) (Choi et al. 1994; Creelman et al. 1992)

and those involved in phenolics such as PPOs (Doares et al. 1995), which are involved in plant defense. Besides, JA provide resistance against root pests like *Bradysia impatiens*, as is clear from the *Arabidopsis thaliana* strains which are deficient in JA and sensitive to this fungus (McConn et al. 1997). It was also observed that the survival of fungus gnat was poor in MeJA-treated plants of spinach (*Spinacia oleracea*) compared to the control (Schmelz et al. 2002). Exogenously applied jasmonates have been found to increase the resistance of *Nicotiana* species against the pests such as tobacco hornworm (*Manduca sexta* L.). Plants of *Nicotiana attenuata* treated with MeJA significantly reduced both florivory and herbivory (McCall and Karban 2006). During herbivore feeding jasmonates induce the synthesis of innumerable volatile compounds in plants, which are similar in response. These include indole, (Z)-3-hexenyl acetate, (E)-β-ocimene, and (E, E)-α-farnesene (Rodriguez-Saona et al. 2001). In *Zea mays* there was local emission of terpenes by the application of MeJA (Farag and Pare 2002). Recently in tomato (*Solanum lycopersicum*), with the help of jasmonate biosynthetic mutant, it has been demonstrated that jasmonate signaling prevents herbivory by the emission of volatile compounds. Tomato plants which are JA-deficient exhibit reduction in volatile emissions and show susceptibility for *Bemisia tabaci* and *Manduca sexta* for oviposition (Sanchez-Hernandez et al. 2006). Phytoalexins are produced in plants like *Arachis hypogaea* (Chung et al. 2003) and *Cupressus lusitanica* (Zhao et al. 2001, 2004) in response to JA, but phytoalexins were not detected in *Solanum lycopersicum*, *S. tuberosum* (Cohen et al. 1993), and *A. thaliana* (Thomma et al. 1999). Defense against pathogens induced by jasmonates shows promising potential for the improvement of crops in horticulture (Pena-Cortes et al. 2005). For instance, JA signaling pathway was required to provide resistance against root-rot diseases caused by *Pythium irregulare* (Staswick et al. 1998) and *Pythium mastophorum* (Drechs.) (Vijayan et al. 1998) in *Arabidopsis thaliana*. In this chapter, we have discussed the role of the JA in the plant signaling mechanism which ultimately activates the responses of plants to different stresses and various kinds of physiological and biochemical activities. We have also focused on the mechanism of jasmonate signaling and the various components involved in it. Additionally, it has been highlighted as to how the jasmonate signaling pathway shows molecular cross talk with the signaling pathways of various plant hormones like auxins, gibberellins, salicylates, ethylene, and abscisic acid.

15.2 Mechanism of Jasmonate Signaling

Jasmonate signaling is thought to be induced by different abiotic stresses, which may include wounding, drought, and exposure to "elicitors" like chitins, oligosaccharides, oligogalacturonides (Doares et al. 1995), osmotic stress (Kramell et al. 1995), salt stress (Dombrowski 2003; Pedranzani et al. 2003), UV irradiation (Demkura et al. 2010), ozone atmosphere (Rao et al. 2000), and to extracts from the yeast (Leon et al. 2001). Following herbivore attack jasmonates lead to the induction of both indirect (volatile emissions) and direct (defense proteins) defense mechanisms (Halitschke and Baldwin 2004; Howe 2004; Pieterse et al. 2006). Proteinase inhibitors (PINs), a category of defense proteins in tomato, prevent degradation of proteins in the midgut of herbivores; other proteins induced by herbivores in tomato include leucine aminopeptidases, threonine deaminase (TD), and arginase (Walling 2000; Chen et al. 2004). Our present understanding of the jasmonate signaling comes largely from the studies conducted on *Arabidopsis* mutants which lack jasmonate biosynthesis or jasmonate perception (Mandaokar et al. 2006). Coi 1 is the mutant of *Arabidopsis* which does not show jasmonate responses and is believed to encode an F-box protein (Feys et al. 1994; Xie et al. 1998). Considering this fact in mind, it was suggested that jasmonate signaling and responses are mediated by the actions of Skp-Cullin-F-box complex (SCF^{COI1}), which is a type of E3 ubiquitin ligase protein (Deshaies 1999). It is therefore suggested that jasmonate signaling involves

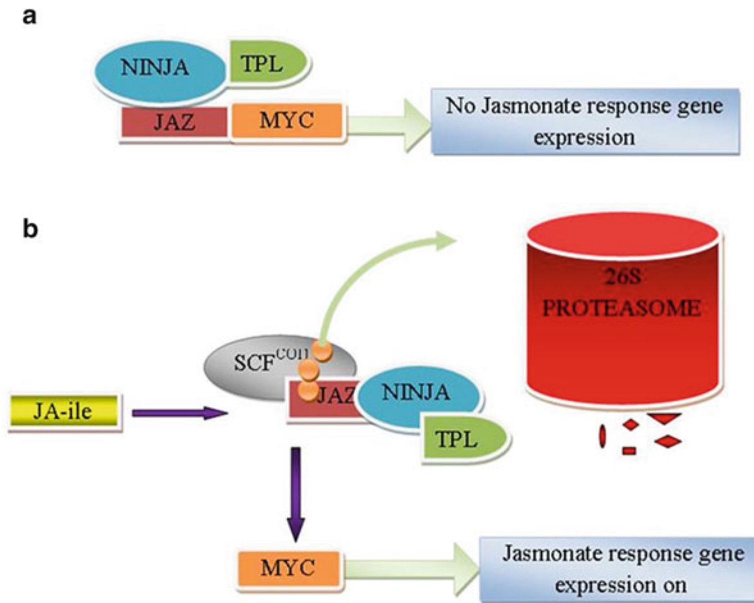

Fig. 15.1 (**a**) In the absence of the active JA-Ile, JAZ repressors recruit corepressors like TOPLESS (TPL) and NINJA which bind transcription factors like MYC. (**b**) In the presence of JA-Ile, JAZ repressors are ubiquitinated by E3 ubiquitin ligase SCF^{COI1} and degraded in the 26S proteasome releasing TFs (MYC) from inhibition and activating JA-responsive gene transcription

ubiquitination of specific target proteins by the SCF^{COI1} complex and their subsequent degradation by a 26S proteasome (Turner et al. 2002). Jasmonates are rapidly synthesized through oxylipin biosynthetic pathway when there is an attack from pathogens or insects (Wasternack 2007; Gfeller et al. 2010). With JA-conjugate synthase (JAR1) jasmonates can readily be conjugated with the isoleucine (Staswick and Tiryaki 2004), resulting in a highly biologically active specific enantiomer, the jasmonoyl-isoleucine (JA-Ile) (Fonseca et al. 2009). The F-box protein coronatine-insensitive 1 (COI1) functions as a JA-Ile receptor in the E3 ubiquitin-ligase Skip-Cullin-F-box complex SCF^{COI1} (Yan et al. 2009; Sheard et al. 2010). Binding of JA-Ile to COI1 leads to degradation of jasmonate transcriptional repressor proteins, Zim-domain (JAZ), via 26S proteasome (Chini et al. 2007; Thines et al. 2007). Consequently, the physical interaction of JAZ proteins with transcriptional activators, leading to repression of jasmonate signaling in resting cells, is broken in JA-stimulated cells, which results in the activation of sufficient JA-responsive genes (Chini et al. 2007; Memelink 2009) as depicted in Fig. 15.1.

15.3 JAZ Proteins

The signaling cascade triggered by JA involves JAZ repressor proteins. The JAZ repressor proteins are degraded by 26S proteasome which releases the transcription factors from the repression, and ultimately their downstream responses are activated. The actual target of SCF^{COI1} complex are the JAZ proteins; it was validated by their COI1-dependent degradation and the mapping of the COI1 locus. Furthermore, from the genetic, molecular, and biochemical studies, it was proved that (1) SCF^{COI1} complex degrades JAZ proteins in the presence of jasmonates; (2) the active form of JA is the jasmonoyl-L-isoleucine (JA-Ile), which is an essential determinant of JAZ protein and the COI1; and (3) the JAZ proteins block the activity of the positive regulator of JA signaling, MYC2 (Chini et al. 2007; Thines et al. 2007; Yan et al. 2007). When

bioactive jasmonates are absent, the activity of MYC2 is blocked by JAZ proteins. JAZ proteins are known to recruit corepressors like TOPLESS (TPL) and TPL-related proteins through the interaction with Novel Interactor of JAZ (NINJA) (Pauwels et al. 2010). MYC2, a basic helix-loop-helix (bHLH) protein, is the first transcription factor which is regulated by JAZ proteins, and its importance in jasmonic signaling was revealed through the forward genetic approach (Lorenzo et al. 2004). MYC2 acts as both positive and negative regulator of jasmonic acid responses; it positively regulates primary root growth inhibition, tolerance against oxidative stresses, and biosynthesis of anthocyanins while it negatively regulates the jasmonate-dependent resistance against necrotrophic fungi, tryptophan, and indole glucosinolate biosynthesis (Lorenzo et al. 2004; Dombrecht et al. 2007). MYC2 can be activated by JA-Ile-induced and SCF^{COI1}-dependent JAZ protein degradation. This model of jasmonate perception and signaling has been discussed by various authors (Memelink 2009; Gfeller et al. 2010).

15.3.1 Structure of JAZ Proteins

JAZ proteins belong to the TIFY group which also includes the proteins Zinc-Finger Expressed In Inflorescence Meristem (ZIM), Zim-like 1 (ZML 1), Zim-like 2 (ZML 2), PPD1, PPD2, and PEAPOD proteins as well as the TIFY8 protein (Vanholme et al. 2007; Bai et al. 2011). JAZ proteins have high sequence variability, but they possess three highly conserved domains which is their distinguishing character. The *N*-terminal (NT) region contains a weakly conserved NT domain, while as the ZIM domain consists of the 30 amino acids within the central portion of the JAZ amino acid sequence which contains a highly conserved TIFY motif (TIF[F/Y] XG) (Vanholme et al. 2007) which provides repressor activity to the JAZ proteins and the formation of homo- and heterodimers within the JAZ family (Chini et al. 2009; Chung and Howe 2009; Pauwels et al. 2010). The *C*-terminal domain (jas domain) is highly conserved throughout the JAZ family with 12 of 29 amino acid residues identical or with conservative substitutions across all 12 *Arabidopsis* JAZ proteins (Chini et al. 2007; Thines et al. 2007). The jas domain mediates protein-protein interactions (Chini et al. 2009; Hou et al. 2011; Song et al. 2011; Zhu et al. 2011) and contains within it a degron which is responsible for the degradation of the JAZs in the presence of JA-Ile (Chini et al. 2007; Thines et al. 2007; Melotto et al. 2008) and is also known to play a role in nuclear localization (Thines et al. 2007; Grunewald et al. 2009).

15.3.2 JA-Ile Mediates Binding of JAZ Proteins to COI1

The physical interaction between COI1 and JAZ1 proteins is mediated by jasmonoyl-isoleucine (JA-Ile), but other derivatives of jasmonates such as 12-oxo-phytodienoic acid (OPDA) and MeJA do not show this property. JA-Ile was found to promote the COI1-JAZ1 interaction in a dose-dependent manner with the stimulatory effect apparent at concentrations as low as 50 nM JA-Ile. Jasmonoyl-isoleucine mediates interaction between COI1 and JAZ1 in yeast cells, which (yeast cells) express COI1 and JAZ1 (without other plant proteins) and suggest that the complex of COI1 and JAZ1 is the perception site for JA-Ile. In auxin signaling the transcriptional repressors, Aux/IAA are removed through SCF-dependent ubiquitination, which involve about six F-box proteins homologous to COI1, and one of the F-box proteins, TIR1, was shown to be an auxin receptor (Tan et al. 2007).

15.3.3 The COI1-JAZ Receptor Complex

Structural and pharmacological studies have shown that the receptor for jasmonates in *Arabidopsis* consists of a COI1 and JAZ complex in which COI1 has a pocket which recognizes (3R,7S)-jasmonoyl-l-isoleucine (JA-Ile). In addition to the above two components, a third component of the jasmonate co-receptor complex has

been found that is called as inositol pentakisphosphate which is known to interact with both COI1 and JAZ components.

15.3.4 Ubiquitin-Proteasome Pathway and Removal of JAZ Repressors

Selective removal of the short-lived regulatory proteins controls the growth and development of plants to a great extent, and one of the pathways for removing such regulatory proteins is the ubiquitin proteasomal pathway. It involves ubiquitin (Ub) and the 26S proteasome, which together form protease complex. Ub gets linked to the protein which is to be degraded, and the resulting complex of Ub-protein is labeled so as to be degraded by the 26S proteasome. Ubiquitin-proteasome pathway (UPP) involves chronological action of three enzymes (E1, E2, and E3) which link the polypeptide, Ub, onto proteins to subject them for degradation (Glickman and Ciechanover 2002; Pickart 2004). This labeling process causes degradation of the ubiquitinated proteins to small peptides as they are recognized by the 26S proteasome which is a multicatalytic protease complex in itself (Baumeister et al. 1998). E1 is the ubiquitin-activating enzyme and E2 is its carrier, and both of these make the ubiquitin ready for the conjugation. The main enzyme involved in the pathway is the E3 (ubiquitin ligase) which detects the particular substrate (protein) and catalyzes the transfer of activated Ub to it. The 26S proteasome is a 2-MDa ATP-dependent proteolytic complex that degrades Ub conjugates; it has been worked out in *Arabidopsis* and rice. When jasmonate signal is absent, JAZ1 and JAZ13/JAZ3 are known to block the activity of JIN1/MYC2 in the nucleus because both JAZ repressors and MYC2 occur in the nucleus at the same time (Lorenzo et al. 2004; Chini et al. 2007; Thines et al. 2007). When the jasmonate signal is present, JAZ repressors are presented to the SCF-E3 complex for ubiquitination and subsequent degradation by the 26S proteasome and by removing these repressors, the activity of JIN1/MYC2 is switched on to control the expression of jasmonate genes.

15.4 Cross Talk of Jasmonate Signaling with Other Plant Hormones

Jasmonates are the phytohormones which elicit the synthesis of secondary metabolites in plant systems. A common example is the tobacco (*Nicotiana tobaccum*) in which the biosynthesis of jasmonates occurs in reaction to herbivore attack stimulating the production of pyridine alkaloids like nicotine (Goossens et al. 2003; Kessler et al. 2004). However, jasmonates do not operate alone; rather they function in multifarious cross-talk network with other phytohormone signaling pathways. Jasmonates are also one of the key hormones which evoke the responses of plant against biotic stresses, though they act in coordination with the signaling pathways of other hormones like salicylates, ethylene, abscisic acid, auxins, and gibberellins in order to ensure a timely, spatially, and fitness-cost correct response (Bari and Jones 2009; Grant and Jones 2009; Pauwels et al. 2009; Kazan and Manners 2008).

15.4.1 Cross Talk of Jasmonates with Salicylic Acid Signaling

JA signaling pathway is known to antagonize salicylic acid (SA) signaling pathway and is evident from the mutant studies of *Arabidopsis* which revealed that the mutations which disturb jasmonate signaling (e.g., *coi1*) cause increase in the PR1 marker gene of salicylic acid, and the mutations which upset SA signaling (e.g., *npr1*) caused parallel rise in the JA marker gene *PDF1.2*. Mur et al. (2006) have shown that the two pathways show dose-dependent interaction because when SA was used exogenously at low concentration, it promoted the induction of *PDF1.2;* however, induction of *PDF1.2* by jasmonates is reduced by using higher SA concentrations. Jasmonate-dependent defenses are found to be effective against necrotrophic

pathogens, but when the plants were given SA and inoculated with active strain of *Pseudomonas syringae*, they show little opposition against necrotrophic pathogens like *Alternaria brassicicola*. However, when the plants were inoculated with avirulent strain of *P. syringae,* the resistance against *Alternaria brassicicola* was not challenged. This leads to the conclusion that SA and jasmonate signaling cross talk is also pathogen specific. Noninducible PR1 (NPR1) is the main component of SA signaling and is also known to mediate the antagonism between salicylic acid and jasmonate signaling to some extent (Spoel et al. 2007). The involvement of NPR1 in SA-JA antagonism is similar to its animal homolog Ikβ, a transcriptional repressor which inhibits prostaglandin synthesis. NPR1 is known to lower down salicylic acid biosynthesis in tobacco plants attacked by insects, which causes concomitant increase in jasmonate biosynthesis and ultimately provides insect resistance (Rayapuram and Baldwin 2007). Another important transcription factor involved in the antagonistic interaction of SA and JA signaling pathways is WRKY70; its overexpression causes continuous expression of the salicylic acid-responsive genes which increases in the immunity to SA-sensitive pathogens but reduces immunity to JA-sensitive pathogens and vice versa (Li et al. 2004). As per Wang et al. (2006) has reported when the level of SA is high, WRKY70 leads to suppression in the level of SA. WRKY62 is also known to negatively regulate JA signaling similar to NPR1 (Mao et al. 2007). One of the positive regulators of JA signaling is MPK4, but it negatively regulates SA signaling. One of the mutants of *Arabidopsis* mpk4 shows constitutive expression of salicylic acid-mediated defense reactions, for example, increase in the level of salicylic acid, continuous production of PR1 defense proteins, and increased resistance to *P. syringae* in the absence of pathogen attack. However, this mutant was found to be lacking in JA-dependent induction of the *PDF1.2* gene. Overexpression of the MKS1, the substrate for MPK4, regulates SA signaling by interacting with the WRKY transcription factors. Glutaredoxin (GRX480) regulates SA-JA interaction in *Arabidopsis* and is known to interact with the TGA-type transcription factors which regulate SA-inducible PR genes and block the expression of *PDF1.2* (Ndamukong et al. 2007). Recently ESR/ESP (epithiospecifying senescence regulator) and WRKY53 have been reported to control SA-JA interaction (Miao and Zentgraf 2007).

15.4.2 Jasmonate and Auxin Signaling

Auxin and JA signaling pathways are known to be connected through ARFs (auxin-responsive factors), and reportedly two of them, ARF6 and ARF8, are necessary for the JA biosynthesis and flower fertility. In fact, Nagpal et al. (2005) have reported that the *arf6/arf8* double mutants are defective in auxin signaling in addition to jasmonic acid paucity, unusual flower maturity, and less expression of the genes responsible for JA-biosynthesis genes in flowers; it provides a clue about the interlinking of the two pathways. Mutations in AXR1 (auxin response gene) produce JA-insensitive plants and hence show the interaction between the pathways of the two hormones (Tiryaki and Staswick 2002). Upon interaction of AUX/IAA proteins with the SCF^{TIR1}, they undergo ubiquitination and degradation. AXR1 controls the SCF^{TIR1} activity by modifying the cullin [a family of hydrophobic proteins providing a scaffold for ubiquitin ligases (E3)] by ubiquitin-like protein Nedd8/Rub1 (Schwechheimer et al. 2002; del Pozo et al. 2002). Since cullin is a general component of SCF complexes, the pleiotropic effect of axr1 mutations suggests that this protein also regulates the activity of SCF^{COI1}. Further, AXR1 is known to encode the subunit of RUB1-activating enzyme and regulates the proteolytic activity of SCF complexes; it is known to regulate the activity of both SCF^{TIR1} and SCF^{COI1} complexes which are involved in auxin and jasmonate signaling, respectively (Schwechheimer et al. 2002). Hence, the mutant axr1 is less sensitive to both jasmonate and auxin signaling (Tiryaki and Staswick 2002). Jasmonate stimulates the expression of auxin biosynthesis genes

(Dombrecht et al. 2007), and likewise auxin activates the expression of JA-biosynthesis genes (Tiryaki and Staswick 2002).

15.4.3 Cross Talk Between Jasmonate and Ethylene Signaling

JA signaling is known to interact with the ethylene signaling both synergistically and antagonistically. JA and ethylene signaling act synergistically to induce the activation of *PDF1.2* gene which in turn provides defense against the pathogens. Induction of *PDF1.2* gene in response to *Arabidopsis brassicicola* requires the synergism between JA and ethylene signaling pathways (Penninckx et al. 1998). In response to biotic stress *PDF1.2* is activated in *Arabidopsis* while its activation is blocked in both ethylene (response) mutant *ein 2* (ethylene-insensitive 2) and jasmonate response mutant *coi1-1*. This implies that ethylene and jasmonates cooperate to effect the gene expression (Penninckx et al. 1996). Ethylene and MeJA show synergistic interaction to induce the gene which encodes osmotin in tobacco (Xu et al. 1994). O'Donnell et al. (1996) have reported that when tomato plants were treated with jasmonate but not with ethylene, it resulted in the induction of wound-responsive proteinase inhibitor *Pin 2* gene. However, when such plants were treated with the ethylene biosynthesis inhibitors, it resulted in abolishment of the responsiveness of *Pin 2* gene to wounding and JA treatment. Thus, the two pathways seemed to coordinate with each other for the expression of a *Pin 2* gene in tomato. The cellulose synthase gene CeSA3/CEV1 acts as a negative regulator of these two pathways because the mutant cev1 shows constitutive jasmonate and ethylene responses (Ellis et al. 2002). The ethylene response factor 1 is the positive regulator of both of these pathways and works at the junction of JA and ethylene signaling (Lorenzo et al. 2003). In addition to the synergistic interaction between these two pathways, there is also antagonistic interaction and one of the common examples is the ozone-induced cell death. Jasmonates prevent the ozone-induced oxidative damage and consequently cell death, while ethylene has antagonistic effect which promotes ozone-induced spread of lesion development. For instance, upon the exposure of ozone (O_3), the radical-induced cell death 1 (rcd1) mutants accumulate salicylic acid and ethylene which collectively promote the continuous production of reactive oxygen species (ROS) and ultimately the cell death, whereas MeJA acts antagonistically to ethylene and inhibits lesion spreading and ROS production (Overmyer et al. 2000, 2003; Langebartels and Kangasjarvi 2004).

15.4.4 Jasmonate and Abscisic Acid Signaling Cross Talk

Abscisic acid and methyl jasmonate (MeJA) show both types of interactions, viz., antagonistic and synergistic, in *Arabidopsis.* They induce stomatal closure by activating the production of reactive oxygen species (ROS) and nitric oxide in stomatal guard cells (Munemasa et al. 2007). In response to drought and water deficit conditions, MeJA is accumulated in plants like abscisic acid (Creelman and Mullet 1995). MeJA induces stomatal closure at a particular concentration and time similar to that of abscisic acid-induced stomatal closing as shown in Fig. 15.2. MeJA does not induce the closure of stomata in *coi 1* mutants, while abscisic acid is able to do so in such mutants. Abscisic acid is known to induce biosynthesis of jasmonic acid in *Arabidopsis,* which results in the increased level of JA that is the reason why abscisic acid-treated plants show reduced salicylic acid defense gene expression (Adie et al. 2007).

15.4.5 Interaction of Jasmonates and Gibberellic Acid Signaling

Gibberellic acid (GA) signal is known to be perceived by GID1 (GA-insensitive dwarf1) (Ueguchi-Tanaka et al. 2007). When there is no GA, DELLA proteins bind to a class of basic

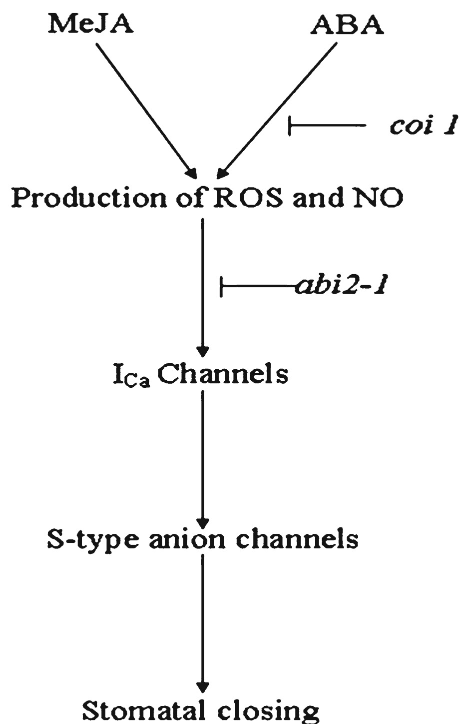

Fig. 15.2 MeJA and ABA mediates closing of stomata through the production of ROS and NO in the guard cells of *Arabidopsis*. *coi 1* mutation disrupts MeJA signaling, while as *abi 2-1* disrupts both (Adapted from Munemasa et al. 2007)

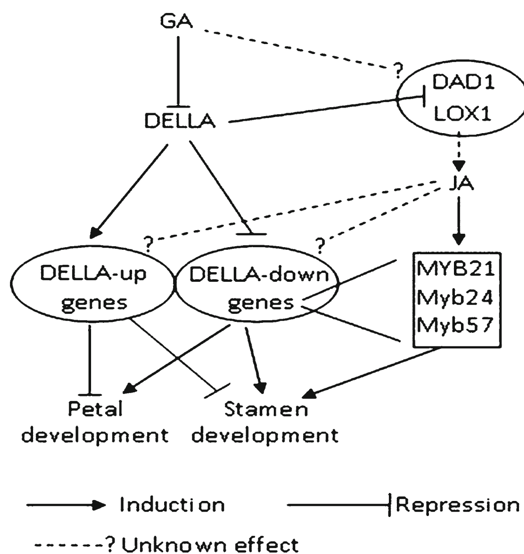

Fig. 15.3 Diagram outlining the cross talk between gibberellin (*GA*) and jasmonic acid (*JA*) during stamen and petal development (From Peng 2009)

helix-loop-helix transcription factors known as phytochrome-interacting factors (PIFs) and inactivate them thereby. When GA is present, there is a structural change in GID1 which binds to DELLA proteins, and the resulting complex (GID1-DELLA) promotes the binding of DELLA to the GID2-SCF complex (Gomi et al. 2004); this ultimately results in triggering of DELLA ubiquitination and degradation. As a result of degradation of the DELLAs, PIFs are released from the repression, thereby promoting the GA-responsive gene expression (Jiang and Fu 2007). DELLAs are also known to interact with the JA signaling pathway. Hou et al. (2010) have demonstrated that DELLAs physically interact with JAZ proteins and prevent AtMYC2 repression and consequently activating the JA signaling. However, in the presence of GA, DELLA proteins are degraded and hence JAZ proteins are active which causes suppression of AtMYC2 expression. Therefore, JA signaling seems to antagonize with the GA signaling and is clear from the DELLA mutants which show increased resistance against biotroph and hemibiotroph pathogens (Navarro et al. 2006). However, in case of stamen development in *Arabidopsis*, the two signaling pathways show synergism. In *Arabidopsis* for the stamen development, three *MYB* (myeloblastosis) genes, namely, *MYB21*, *MYB*24, and *MYB57*, are essential; these genes are regulated by both GA and JA as highlighted in Fig. 15.3. It is confirmed that GA activates the expression of *DAD1* and *LOX1* genes, which are essential for JA biosynthesis. Mutations, which impair filament elongation of the stamen, pollen maturation, or anther dehiscence, result in male sterility in *Arabidopsis*. Deficiency in the plant hormone GA causes male sterility due to accumulation of DELLA proteins, and the presence of GA triggers DELLA degradation to promote stamen development. Likewise GA deficiency of jasmonates is also responsible for the male sterility and is evident from the mutants of these two hormones. For example, biosynthetic mutants of GA (e.g., *ga1-3*) and JA (e.g., *ga1-3*) show male sterility due to failure of stamen filament elongation and of

completion of anthesis and anther dehiscence (Stintzi and Browse 2000; Koornneef and van der Veen 1980).

15.5 Conclusion

Present review provides an overview of the jasmonate signaling in plants and its cross talk with other phytohormones. We have tried to compile the information available about JA signaling and the various components involved in it. Since JA signaling is activated in plants in response to attack by pathogens and under abiotic stresses, efforts to decode JA signaling completely will help to understand plant-to-plant/microbe interactions and also to realize the role of the environment in facilitating their interactions. The role of JA in defense and growth protection promises to pave the way in protection of crop plants and increase the plant productivity.

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16 Salicylic Acid Signaling

Asım Kadıoğlu and Aykut Sağlam

Abstract

Salicylic acid (SA) is a phenolic derivative, found in a wide range of plant species. Signaling role of SA in plants, particularly in defense against pathogens, has only become evident during the past 20 years. In addition to its role in plant defense responses against pathogens, SA has also functions in plant responses to abiotic stress factors such as drought, chilling, heavy metal toxicity, heat, and osmotic stress. During the entire lives of the plants, physiological and biochemical processes including photosynthesis, ion uptake, membrane permeability, enzyme activities, flowering, heat production, and growth and development of plants are regulated by SA. For our better understanding, it is important to know how complex SA signaling works in these physiological processes. Therefore, we need to learn how SA acts and which molecules are related to its roles in plant growth and development. Here, the role of SA during plant growth and development will be discussed.

Keywords

Abiotic stress • Plant growth • Plant metabolism • Salicylic acid

16.1 Introduction

The word salicylic acid (SA) was derived from the Latin word "Salix," meaning willow tree from which SA was isolated as a tiny amount of yellow crystals that was called salicin by John Buchner in 1928. During the following year Henri Leroux had improved the extraction process to obtain 20 % product from 100 g willow. Ten years later in 1938, Raffaele Piria had converted salicin into salicylic acid for the first time (Jeffreys 2008).

A. Kadıoğlu (✉)
Department of Biology, Faculty of Sciences, Karadeniz Technical University, 61080 Trabzon, Turkey
e-mail: kadioglu@ktu.edu.tr

A. Sağlam
Department of Molecular Biology and Genetics, Faculty of Sciences, Karadeniz Technical University, 61080 Trabzon, Turkey

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Classified under the group of plant hormones, salicylic acid is a phenolic compound that has been characterized in many plants, belonging to diverse groups. SA levels of these species are widely different (Rivas-San Vicente and Plasencia 2011). For instance, SA level in the model plant *Arabidopsis thaliana* is ranging from 0.250 μg to 1 μg g^{-1} FW (Nawrath and Metraux 1999). In addition, the SA level can also be quite different within members of the same family. For example, in the Solanaceae family, whereas tobacco leaves (*Nicotiana tabacum*) contain <100 ng g^{-1} FW (Malamy et al. 1992), potato (*Solanum tuberosum*) contains up to 10 μg of total SA g^{-1} FW (Navarre and Mayo 2004).

Chemically, SA belongs to plant phenolics and is derived from an intermediate in the shikimic acid pathway, cinnamic acid. Two pathways were proposed for its synthesis. In the first route cinnamic acid is converted to benzoic acid by decarboxylation then SA is synthetized from benzoic acid enzymatically. This route is proposed in tobacco, rice, and *Quercus pedunculata* (Alibert and Ranjeva 1971; Yalpani et al. 1993; Silverman et al. 1995). In the second route, cinnamic acid is converted into o-coumaric acid by trans-cinnamate-4 hydroxylase, and then *o-coumaric* acid is decarboxylated to form SA (Alibert and Ranjeva 1971, 1972).

In plants, SA is transported, metabolized, and conjugated. Most of SA synthesized in plants is found as glucosylated and/or methylated forms. Conjugation reactions occur in the cytosol. The major glucose conjugate is the SA glucoside [SA 2-*O*-β-D-glucoside] that is formed by conjugating at the hydroxyl group of SA, while SA glucose ester is formed after conjugation at the SA carboxyl group. SA glucosyltransferases catalyze conjugation reactions (Song 2006). In soybean and tobacco cells, SA glucoside is actively transported from the cytosol into the vacuole. It may function in the vacuole as an inactive storage form, which can release free SA (Dean and Mills 2004; Dean et al. 2005). In addition, SA carboxyl methyltransferase catalyzes conversion of SA to methyl salicylate (MeSA), an important long-distance signal, which functions in systemic acquired resistance of *Arabidopsis thaliana* and tobacco (Shulaev et al. 1997; Chen et al. 2003). Glycosylation of MeSA produces MeSA 2-*O*-β-d-glucose that is not stored in the vacuole (Dean et al. 2005).

SA, assigned diverse regulatory roles in the metabolism of plants, is accepted as a signal molecule in responses of the plants to abiotic stresses such as drought, chilling, heat, and osmotic stress (Borsani et al. 2001; Kang and Saltveit 2002; Chini et al. 2004; Larkindale et al. 2005). On the other hand, the role of SA in local and systemic response against microbial pathogens has also been well documented (Durrant and Dong 2004; Vlot et al. 2008).

This chapter summarizes the recent advances in the understanding of the physiological functions and the role of SA in plant growth and development in particular, and its effects on seed germination, vegetative growth, photosynthesis, stomatal closure, thermogenesis, nitrate metabolism, nodulation in roots, and flowering.

16.2 Physiological Roles of Salicylic Acid

16.2.1 Effects of SA on Seed Germination

Seed germination can either be increased or decreased by SA. These contradictory reports on the role of SA in seed germination are related to SA concentrations. For instance, SA doses ranging from 3 to 5 mM can inhibit maize germination (Guan and Scandalios 1995). Germination of *A. thaliana* seeds can be delayed or inhibited by SA concentrations higher than 1 mM (Rajjou et al. 2006). In barley, doses higher than 0.25 mM SA inhibit seed germination (Xie et al. 2007). Negative effects of SA on the seed germination are presumably due to a SA-induced oxidative stress. A threefold increase in hydrogen peroxide content was determined in *Arabidopsis* plants treated with SA (1–5 mM) as compared to control. In addition to increase in H_2O_2, increase in Cu- and Zn-superoxide dismutase and inactivation of the catalase and ascorbate peroxidase were also determined (Rao et al. 1997).

During germination of seed, a cross talk among SA and both ABA and GAs was indicated in previous studies. GAs have a role in the SA biosynthesis in *Arabidopsis*. Exogenous GA application (50 μM) to Arabidopsis seeds results in increase in GA-stimulated gene introduced to *Arabidopsis* from beechnut (*FcGASA*$_4$) and endogenous SA content up to twofold compared to non-GA-treated plants (Xie et al. 2007). In addition, *FcGASA*$_4$-overexpressing *Arabidopsis* lines grown in the presence of GA_3 showed induced expression of the SA biosynthesis and perception genes, *ICS1* (isochorismate synthase) and *NPR1* (nonexpressor of *PR-1*), respectively. Under 150 mM NaCl stress, seed germination of *sid2* (SA-induction deficient 2) mutant that is of defects in SA synthesis was improved by a 50 μM GA3 application. On the other hand, seed germination of the *A. thaliana ga1-3* mutant (GA-deficient) was rescued by 50 μM of exogenous SA treatment (Alonso-Ramirez et al. 2009). Although the synergistic effects of SA with GA on seed germination were reported. They had also antagonistic effects. For instance higher-dose SA inhibited seed germination of barley plants by suppressing GA-induced α-amylase (*Amy32b*) expression through induction of a WRKY repressor (HvWRKY38) which is down-regulated by GAs, whereas upregulated by SA and ABA (Xie et al. 2007). In *Arabidopsis* seeds, synthesis of some ABA-regulated proteins such as embryogenesis abundant (LEA) proteins, dehydrins, and heat shock proteins in the presence of 0.5 mM SA was a proof for cross talk between SA and ABA (Rajjou et al. 2006).

16.2.2 Effect of SA on Plant Growth

Plant growth responses to SA depend on species and developmental stage, and the SA concentration used. Plant growth-improving effects of exogenous SA were reported for some plants such as soybean, wheat, maize, and chamomile (Gunes et al. 2007; Kovacik et al. 2009). Increases in shoot and root growth were determined in soybean plants that were subjected to SA ranging from 10 nM to 10 mM SA, 7 days after application. On the other hand, more evidences coming from mutant or transgenic *Arabidopsis* plants affected in SA signaling showed that endogenous SA had key roles in plant cell growth and it might be a negative regulator of the cell growth. For instance, *Arabidopsis thaliana* overexpressing SA-induced DNA binding with one finger transcription factor OBP3 (OBF4 Binding Protein 3) showed a decreased growth rate in roots and leaves (Kang and Singh 2000). In addition, Arabidopsis *cpr5* (constitutive expressor of PR5) and *agd2* (aberrant growth and death) mutants having high levels of SA exhibited dwarf phenotypes (Bowling et al. 1997; Rate and Greenberg 2001). Adversely, the growth rate of SA-deficient Arabidopsis *NahG* mutant was 1.7-fold higher than wild-type plant (Du et al. 2009).

Enhanced cell division in roots of wheat seedlings was reported after 50 μM of SA application (Shakirova et al. 2003). Similarly, the growth of leaf rosettes and roots of chamomile plants was induced by 50 μM SA, but 250 μM SA suppressed growth of these organs (Kovacik et al. 2009). Increase in leaf area and dry matter production in SA, acetylsalicylic acid (ASA), and an SA analog gentisic acid (GTA)-treated soybean and corn plants was determined (Khan et al. 2003). Fariduddin et al. (2003) reported that SA treatment (10^{-5} M) to *Brassica juncea* resulted in a maximum increase in dry matter accumulation in the leaves, whereas, higher SA concentration had inhibitory effects on the plant growth. Moreover, Hayat et al. (2005) recorded that wheat seedling grown from the seeds that imbibed in 10^{-5} M of SA had more leaves and higher fresh and dry weights than non-SA-treated plants. Likewise, SA enhanced growth of barley seedlings and delayed leaf forming. Leaves of SA-treated seedlings reached maturity later than the control group, and their leaf blades become more narrow and shorter (Pancheva et al. 1996). These differences in barley responses suggest that effects of SA on plant growth are dose dependent. Similarly, the SA effect on bud formation of *Funaria hygrometrica* was in a dose-dependent manner that high concentration of SA inhibited bud formation while lower doses induced it (Christianson and Duffy 2002).

16.2.3 Effect of SA on Photosynthesis and Stomata

The effects of exogenous SA on the photosynthetic processes are well documented (Horvath et al. 2007). For example, 30-day-old mustard plants sprayed with a low concentration of SA (10^{-5} M) acquired a higher net photosynthetic rate, which was also reflected in the form of healthy growth of the plants and increased chlorophyll content and dry mass production (Fariduddin et al. 2003); however, the chlorophyll contents of the plants treated with 10^{-3} M decreased and the values were below that of control, at a 60-day period.

The photosynthetic electron transport rate in wheat was elevated by SA in chloroplasts (Sahu et al. 2002). Harmful effects of paraquat and cadmium on barley and maize photosynthesis were alleviated by exogenous SA treatment (Ananieva et al. 2002; Krantev et al. 2008). In wheat and tomato, SA reversed salt stress-induced inhibition of plant growth and increased net photosynthesis (Arfan et al. 2007; Poor et al. 2011). In addition, SA (20 mg ml^{-1}) increased chlorophyll contents of *Brassica napus* leaves (Ghai et al. 2002). Hayat et al. (2005) reported that pigment content increased in leaves of wheat grown from the seeds soaked in 10^{-5} M SA. However, little is known about SA-related mechanisms that alleviate the decline of photosynthesis. On the other hand, high concentrations of SA may also be toxic (Uzunova and Popova 2000). Chlorophyll and carotenoid contents of *Vigna mungo* seeds that were soaked in different SA solutions, which is ranging from 10 to 150 μM, decreased, whereas the control group that was irrigated immediately after SA treatments was not affected as much as the group not irrigated after SA application (Anandhi and Ramanujam 1997). Similarly, a decrease in chlorophyll content in the leaves of barley plants grown in culture medium with SA (0.1–1 mM) was detected as compared to control (Pancheva et al. 1996). Carotenoid and xanthophyll synthesis and de-epoxidation rates of wheat and moong plants that were treated with salicylic acid increased, while chlorophyll contents and chlorophyll a/b ratio decreased in both plants after SA treatment (Moharekar et al. 2003). Moreover, SA-treated *Spirodela* has less anthocyanin and chlorophyll pigments than non-treated plants (Khurana and Maheshwari 1980). Because of the fact that SA is of inhibitory effects, it is important to know how SA affects leaf metabolism, especially photosynthetic functions, and whether these effects are direct or indirect ones for increasing stress tolerance. Effects of SA are not only related to plant species but also the mode of SA application (soaking, spraying, adding to growth media, etc.) and the longevity of the treatment. SA has not only adverse effects as mentioned above. Exogenous SA that was taken up by plants and transported within the plants may also enhance endogenous SA synthesis. In maize, *Ctenanthe setosa*, and *Phlox* plants, exogenous SA treatments increased endogenous SA levels (Talieva and Kondrat'eva 2002; Kadioglu et al. 2011; Demiralay et al. 2013). According to the majority of the results, described effects of the SA treatments on plants are not only related to SA itself but also due to indirect ones induced by the treatment at the site of SA application.

SA has also effects of Calvin cycle enzymes such as PEPCase and RuBisCo. However, the reports about the SA effects on these enzymes are contradictory. Barley plants exposed to SA ranging from 0.1 to 1 mM for 1 week had about 50 % low ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCo) activity compared to control (Pancheva and Popova 1998). Pancheva and Popova (1998) also reported an increase in PEPCase in barley and a decline in the photosynthetic rate. On the other hand, SA treatment increased RuBisCo activity and photosynthesis rate in maize plants (Khodary 2004). Fariduddin et al. (2003) recorded increased water use and carboxylation efficiencies in association with high photosynthetic rate in mustard plants.

Stomatal closure regulated by plant hormones is an important factor for photosynthesis (Acharya and Assmann 2009). It has been proved that SA is one of the regulatory phytohormones for stomatal closure by recent studies (Melotto et al. 2006). SA-induced stomatal closure in *Arabidopsis* subjected to 400 μM of SA was determined at 2 h after SA treatment (Mateo et al. 2004). Similarly, Larque-Saavedra (1979)

determined SA-induced stomatal closure and decreased transpiration in French bean. On the other hand, SA-applied soybean plants exhibited high internal CO_2 concentration, water use efficiency, and transpiration rate (Kumar et al. 2000). Similar to soybean, maize and barley plants that were treated with SA had high stomatal conductance and transpiration rate (Pancheva et al. 1996; Khan et al. 2003).

16.2.4 Effects of SA on Leaf Rolling

Leaf rolling represents a dynamic behavioral response in plants in which sunlight orientation of the two leaf surfaces may be reversed (Smith 2008). Light interception, transpiration, and leaf dehydration are reduced during leaf rolling playing a role in osmotic adjustment to maintain internal plant water status (Subashri et al. 2009). On the other hand, delayed leaf rolling indicates the ability of leaf to sustain turgor through increased water uptake or osmotic adjustment despite drought stress. In addition, the leaves are protected from the effects of excess radiation by leaf rolling (Kadioglu and Terzi 2007).

Leaf rolling increases photosynthesis under mild water stress (Lang et al. 2004); however, under mild water stress, photosynthesis and yield may also decrease during leaf rolling due to reduced light absorption by the photosynthetic system (Jones et al. 1998; Kadioglu et al. 2012). Under these water stress conditions, delay in the leaf rolling could be beneficial for plants. Therefore, some studies to find out possible ways that could retard leaf rolling have been conducted (Kadioglu et al. 2012). Salicylic acid has been shown to delay leaf rolling due to the prevention of water loss and induction of antioxidant enzymes in mature maize (Saruhan et al. 2012) and in *Ctenanthe setosa* plants (Kadioglu et al. 2011). Demiralay et al. (2013) claimed that SA might have hydrogen peroxide-mediated regulatory role on leaf rolling by inducing antioxidant enzymes. The induced antioxidant enzymes reduced photooxidative damage that we resulted from the excess accumulation of hydrogen peroxide and then delayed leaf rolling.

16.2.5 Effects of SA on Nodule Formation

In the case of symbiotic bacteria in the genera *Rhizobium*, *Bradyrhizobium*, *Allorhizobium*, *Mesorhizobium*, and *Sinorhizobium*, a beneficial relationship is established that results in nodule formation and atmospheric nitrogen fixation instead of a defense response. By this relationship, the host plant supplies a protected environment and carbon sources to the bacteria. As to bacteria, they fix atmospheric nitrogen that plants can use (Stacey et al. 1995). SA can also affect the interaction of plants with symbiotic microorganisms. For example, *Sinorhizobium meliloti* nodC mutant that can't synthesize lipochitin Nod signal for infection caused an increase in SA accumulation in alfalfa roots (Martínez-Abarca et al. 1998). On the other hand, nodule formation in alfalfa roots inoculated with wild-type *S. meliloti* was reduced and delayed by exogenous SA treatment (Martínez-Abarca et al. 1998). In addition, persistent nodule formation in *Vicia sativa* was inhibited by exogenous SA treatment (van Spronsen et al. 2003). They claimed that lipochitin nodulation signal produced by *Rhizobium leguminosarum* bv. *viciae* that nodulates *Vicia* was inhibited by SA. Another study to show the impact of SA on nodule formation was conducted by Stacey et al. (2006). Growth of bacterial symbiont was reduced and nodulation was inhibited by exogenous SA treatment. They also indicated that endogenous SA was also important for SA effects on nodule formation. They showed that nodulation and infection increased in the roots of a salicylic acid mutant *NahG* of *Lotus japonicus* and *Medicago truncatula* plants that have reduced SA.

16.2.6 Effect of SA on Nitrogen Metabolism

Plants tend to maintain constant levels of essential nutrients to continue optimal growth and development; however, availability of nutrients in the soil is limited and they are found in low concentrations (Schachtman and Shin 2007). Plants have some mechanisms to cope with reduced nutrient

availability; thus, they increase nutrient acquisition (Lopez-Bucio et al. 2003; Svistoonoff et al. 2007). Plant productivity was decreased due to low availability of ammonium and nitrate in soil; however, plants grown under low N supply were evolved to acquire more soil N. Their roots produce lateral roots to increase the surface area that contact more N in soil. This resulted in increased amount of N uptake (Lopez-Bucio et al. 2003; Bloom et al. 2010). Despite these responses are helpful at maturity, but these morphological changes during germination and seedling growth cannot be sufficient. Therefore, salicylic acid is drawn attention as phenolic acids based on regulation of nitrogen metabolism (Ramirez et al. 2009). The role of SA on nitrogen metabolism was reviewed. For instance, Jain and Srivastava (1981) found that SA in association with $Ca(NO_3)_2$ increased nitrogen uptake and nitrate reductase (NR) activity in the roots and leaves of maize plants. Similarly, Rane et al. (1995) recorded that NR activity in the presence of NO_3 increased and enzyme protected against trypsine (proteinase) by SA application. In addition, increased NR activity was determined in wheat seeds soaked in 10^{-5} M of SA (Hayat et al. 2005). The activity increased by 36 % compared to control. SA (10^{-5} M) treatment increased NR activity by 13 % in mustard leaves compared to control (Fariduddin et al. 2003). SA also induced NR activity and protein content in soybean (Kumar et al. 1999); however, a decrease in soluble protein content in SA-treated barley was determined (Pancheva et al. 1996). Similar to proteins, SA application decreased the level of sugars, starch, and phenols in *Vigna mungo* cultivars (Anandhi and Ramanujam 1997).

16.2.7 Effect of SA on Respiration and Heat Production

A cyanide-resistant "alternative" electron transfer pathway is one of the distinguishing features of plant mitochondria (Moore and Siedow 1991; Siedow and Umbach 1995). To produce water, the alternative oxidase that transfers electrons from reduced ubiquinone to molecular O_2 is the key enzyme of the alternative pathway (Moore and Siedow 1991). Alternative oxidase bypasses proton translocation. The protons are not pumped across the inner membrane by the alternative oxidase; thus, energy is released as heat (Whitehouse and Moore 1995). This pathway found in all higher plant, some algae, and fungi promotes thermogenesis during flowering in aroid spadices (Moore and Siedow 1991). Expression of alternative oxidase was induced by SA in the appendix tissue of voodoo lily (*Sauromatum guttatum*) (Raskin et al. 1987), where SA acts as an endogenous trigger of thermogenesis. Thermogenesis will be discussed later. Alternative oxidase expression in tobacco (*Nicotiana tabacum* L.) was induced by SA application (Rhoads and McIntosh 1993). Moreover, after 4 h of SA application, a two to sixfold increase in *NtAOX1* gene expression in a concentration-dependent manner was determined in tobacco (Norman et al. 2004).

In tobacco cell cultures, SA as low as 20 μM inhibits both within minutes as well, whereas SA higher than 50 μM inhibits significantly ATP synthesis and respiratory O_2 uptake. Decreases in ATP levels by 50 % by SA treatment (500 μM) were determined in tobacco cells grown in a culture media (Xie and Chen 1999). The inhibition in ATP synthesis in the tobacco cells is due to induction of alternative respiration by SA (Kapulnik et al. 1992).

Thermogenesis is linked to cyanide-resistant respiration involving the alternative oxidase pathway (James and Beevers 1950; Meeuse 1975). Heat production usually began first in male flowers and spreads throughout the inflorescence. Salicylic acid "calorigen" triggers an increase in the alternative oxidase and heat evolution in the voodoo lily (Raskin et al. 1987). Inductions in the alternative oxidase gene and protein were reported after SA application (Elthon and McIntosh 1987; Rhoads and McIntosh 1991). Isolated mitochondria of dormant and dormancy-breaking potato tubers were treated with 20 μM SA. Increase in the capacity of cyanide-resistant respiration was determined. In addition, SA enhanced the involvement of the alternative pathway and increased temperature in potato tubers (Wen and Liang 1994). The heat production in plants and the involvement of the SA are well documented (Raskin 1992a). About 1 mg g^{-1} increase in the contents of SA was determined

during heat production in male cones of four thermogenic cycads (Raskin et al. 1990). About 12 °C increase was measured in flowers of lilies that were subjected to 0.13 mg g^{-1} FW of SA (Raskin et al. 1989). Increase between 0.5 and 1.0 °C in the surface temperature of tobacco leaves was measured with a high-resolution infrared camera. Using salicylhydroxamic acid and propyl gallate that were inhibitors of the alternative pathway hindered the thermal effect of SA. Van Der Straeten et al. (1995) suggested that SA increased the activity of total respiration and of the cyanide-resistant pathway in tobacco leaves and then leaf temperature climbed.

16.2.8 Effect of SA on Flowering

It has been proposed that SA might regulate flowering in plants (Goto 1981). SA effects on induction of flowers in tobacco cell culture supplemented with kinetin and indole acetic acid were reported by Eberhard et al. (1989). Similarly, the flowering of *Xanthium strumarium* was stimulated by SA (Cleland and Ajami 1974). SA also accelerated flowering in *Pisita stratiotes*, Arecaceae, grown in culture medium with SA (Piterse 1982). Same responses in flowering come from the studies on *Spirodela polyrhiza* and *Spirodela punctata* (Khurana and Maheshwari 1980; Scharfetter et al. 1978). The synergistic effect of SA with GA was proven by comparing the individual effect of GA and SA on flowering in *Impatiens balsamina* and *Arabidopsis thaliana* (Sood and Nanda 1979; Goto 1981). Oota (1975) hypothesized the free *o*-hydroxyl group on benzoic acid may have a role in flower induction. This was supported by studies using chelating that induce flowering in *Lemnaceae* (Oota 1972) Similarly, Raskin (1992b) reported flower-inducing activity of benzoic acid. Watanabe et al. (1981) showed other non-chelating phenolics could induce flowering and concluded that rather than the general belief, additional mechanisms may be involved in flowering. On the other hand Martinez et al. (2004) reported that late flowering was detected in SA-deficient *Arabidopsis* mutants (*NahG, sid1/eds5, and sid2*). Recently a transcription factor, HAHB10 (*Helianthus annuus* homeobox-10) belongs to the HD-Zip II family, making links between SA and flowering was described in sunflower. This factor induces flowering by upregulating flowering genes (Dezar et al. 2011).

16.3 Conclusion and Future Perspectives

SA contributes to growth and developmental regulation of plants. Biochemical mechanisms that rely on the responses of plants to SA are not known completely, and roles of SA in plant growth and development are still controversial. In addition, the questions of how SA is known and initially perceived by the plant cells, then responses begin, are still being answered. However, various phenotypes having regulated SA levels and mutant studies will enlighten these issues. Understanding the role of SA in stress and plant development will let us to find out the compromise between stress response and growth.

SA interacts with several plant hormones and signaling molecules that have roles in developmental processes of the plants. The important point is to reveal the relationship between SA and hormones and signaling molecules, which affects different stages of plant growth. In addition, the SA-mediated responses that occur in the plant body are related to the developmental state of a plant. Therefore, the response to SA can be different due to plant capacity. Finding answers to these issues will help us to establish a network of relationship between SA and plant growth. Thus, we will develop having more yield and stress resistance.

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Ethylene Signaling in Plants: Introspection

17

Syed Sabhi Ahmad, Waseem Shahri, Shiekh Tajamul Islam, Riyaz Ahmad Dar, and Inayatullah Tahir

Abstract

Ethylene is the simplest unsaturated hydrocarbon gas produced in most plants that regulates a number of biochemical processes. Ethylene regulates a wide array of developmental processes, but its precise role in the regulation of these processes is still not clear. Ethylene's role as a signal molecule depends on the cell response to its changing concentrations and the processing of this information in the form of physiological responses in the target cell. Ethylene is perceived by a family of ER-membrane-bound receptors encoded by the ethylene response 1 (ETR1) gene, and these receptors transduce the ethylene signal. Other ethylene receptors such as ERS1, ERS2, EIN4, ETR1, and ETR2 act as negative regulators via constitutive triple response 1 (CTR1) gene. The CTR1 is presumed to show similarities with Raf, a mitogen-activated protein kinase kinase kinase (MAPKKK) and thus is thought to function like Raf, in a typical MAPK cascade. It has been demonstrated that CTR1 binds ER membrane via ETR1 or by a direct association with ERS1 and ETR2 during ethylene signaling. Ethylene is thought to regulate several aspects of plant growth involving associations with other plant hormones primarily auxins and gibberellins.

Keywords

Ethylene • Ethylene receptors • Ethylene response 1 (ETR1) • Constitutive triple response 1 (CTR1) • Auxin resistant 1 (AXR1)

S.S. Ahmad • W. Shahri • R.A. Dar • I. Tahir (✉)
Plant Physiology and Biochemistry Research Laboratory, Department of Botany, University of Kashmir, Srinagar 190006, Jammu and Kashmir, India
e-mail: syedsabhiindrabi@gmail.com; waseem.bot@gmail.com; reyazadar@gmail.com; inayatullahtahir@gmail.com

S.T. Islam
P.G. Programme in Bioresources, Department of Botany, University of Kashmir, Srinagar 190006, Jammu and Kashmir, India
e-mail: tajamul166@gmail.com

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17.1 Introduction

In all living organisms, cells constantly communicate with each other. In plants, cells communicate to coordinate their activities in response to several internal and external environmental changes like change in temperature, light, and darkness (Alberts et al. 2002; Abeles et al. 1992; Bleecker and Kende 2000; Argueso et al. 2007). To respond to these changes, living organisms harbor diverse and sophisticated signaling strategies (Takeshi et al. 2000). In addition to a vast number of signaling molecules like sugars and protein kinases, various plant growth regulators including auxins, abscisic acid, ethylene, cytokinins, and gibberellins help to coordinate plant growth and development by acting as signaling molecules (Takeshi et al. 2000; Alberts et al. 2002). These growth regulators readily diffuse through cell walls and act in multiple ways inside the cell. The specific effect depends on the stimulus generated by the target cell (Alberts et al. 2002). Hormonal signals combine external environmental inputs and internal developmental and translate them into desirable responses (Alberts et al. 2002; Klee 2004). Plants modulate these signals in multiple ways, and regulation can occur at the level of hormone synthesis, transport, uptake, or turnover or at the level of ethylene perception or signal transduction (Klee 2004). Among these, ethylene acts as a primary mediator to respond to and coordinate internal and external developmental cues in modulating plant growth and developmental processes (Alberts et al. 2002; Takeshi et al. 2000; Yoo et al. 2009; Ju and Chang 2012; Schaller 2012). Ethylene is the simplest unsaturated hydrocarbon gas formed in most plant and cell types and regulates diverse metabolic and developmental cues including agronomically important processes involved from seed germination to organ senescence and abscission, cell elongation, nodulation, pathogen response, and response to abiotic and biotic stresses (Abeles et al. 1992; Theologis 1998; Stepanova and Ecker 2000; Bleecker and Kende 2000; Schaller and Kieber 2002; Tsuchisaka and Theologis 2004; Guo and Ecker 2004; Klee 2004; Chen et al. 2005; Yoo et al. 2009; Ju and Chang 2012; Schaller 2012). A molecular cross talk exists between ethylene signaling pathways and other hormonal signaling molecules particularly auxins, cytokinins, abscisic acid, gibberellins, and brassinosteroids (Chen et al. 2005). The broad spectrum of ethylene effects in plant growth and development has led to its detailed investigation. Significant improvement has been made in understanding the mechanism of ethylene signal perception and transduction (Bleecker and Kende 2000; Stepanova and Alonso 2009; Ju and Chang 2012). In this review, we will focus on the role of ethylene as a signal molecule. Moreover the mechanism of ethylene signal perception and transduction will be dealt with detail.

17.2 Ethylene as a Signal Molecule

Ethylene regulates a wide array of plant processes right from seed germination up to organ abscission or senescence. Ethylene was recognized as a signal molecule with the studies of Abeles et al. (1992). The involvement of ethylene in plant growth and development was first demonstrated by Dimitry Neljubov in 1901 when it was reported that leaks in the illuminating gas caused premature senescence and defoliation of plants in greenhouse and of trees near gas lines (Abeles et al. 1992). Neljubov observed that horizontal growth habit was exhibited by etiolated pea seedlings in the laboratory in contrast to the upright habit shown by the pea seedling when grown in outside air. He later on proposed that the contaminating gas was the causative agent for the abnormal growth habit. This causative agent later on proved to be ethylene gas (Abeles et al. 1992; Bleecker and Kende 2000; Guo and Ecker 2004). This led Neljubov to propose “triple response of ethylene” on etiolated dicotyledonous seedlings. Triple response is characterized by the inhibition of root cell and hypocotyl elongation, curvature of the apical hook, and radial swelling of the hypocotyls. Later on Gane (1934) proposed that ethylene is produced by the plants internally. This proposal laid the stage for investigation of

ethylene as an endogenous signal molecule in plants (Johnson and Ecker 1998; Bleecker and Kende 2000; Alberts et al. 2002). Ethylene is thought to regulate a wide array of developmental processes, but it is still unclear as to how this hormone is concerned with such varied and different functions (Leon and Sheen 2003; Alonso and Stepanova 2004; Yoo et al. 2009). Recent evidences have suggested that plant tissues differ in their sensitivity to ethylene and also with their developmental stage because of the signaling interactions with other plant growth regulators and other metabolites (Leon and Sheen 2003; Alonso and Stepanova 2004; Ramon et al. 2008; Yoo et al. 2009).

17.3 Ethylene Signaling

17.3.1 Ethylene Biosynthesis

Surprisingly, the multitude of physiological responses governed by ethylene signaling is vast, and it exhibits various morphogenetic effects (Abeles et al. 1992; Theologis 1998; Stepanova and Ecker 2000; Bleecker and Ken2000; Wang et al. 2002; Schaller and Kieber 2002; Tsuchisaka and Theologis 2004; Guo and Ecker 2004; Klee 2004; Chen et al. 2005; Yoo et al. 2009; Lin et al. 2009; Ju and Chang 2012; Schaller 2012). This wide range of specificity has been attributed to its complex biosynthetic pathway (Abeles et al. 1992; Kieber 1997; Theologis 1998; Adams and Yang 1979). Ethylene is found to be produced in almost all parts of the higher plants, and the rate of ethylene production varies from one part of the plant to another depending on the stage and type of tissue. Among different tissues, meristems are the most active regions of ethylene biosynthesis. Despite its chemical simplicity, the biosynthesis probably represents the agonies and frustration of the plant scientists who were involved with its study. Ethylene is formed from the precursor methionine (Met) via S-adenosyl methionine (AdoMet) and 1-aminocyclopropane-1-carboxylic acid (ACC) (Yang and Adams). The enzymes AdoMet synthetase, ACC synthase (requires pyridoxal phosphate as cofactor), and ACC oxidase occur in series to produce ethylene from methionine (Kende 1993). During every cycle, 5′-methylthioadenosine is generated which acts as precursor for the formation of methionine which then enters the Yang cycle to produce ethylene again via modified methionine cycle (Miyazaki and Yang 1987). This pathway provides methylthio group for ethylene production at the cost of a single ATP molecule for every cycle. ACC synthase (the main enzyme in the ethylene biosynthesis pathway) shows structural similarity with aminotransferases as11 of the 12 amino acid residues in ACC synthase are conserved in aminotransferases. The expression ACC synthase gene is regulated by various developmental, environmental, and hormonal signals (Zarembinski and Theologis 1994; Bleecker and Kende 2000). Studies on ethylene-related mutants in *Arabidopsis* showed that overproduction of ethylene in these mutants occurs as a result of the elevated ACC synthase activities (Guzman and Ecker 1990; Vogel et al. 1998; Woeste et al. 1999; Bleecker and Kende 2000). Thus, in ethylene biosynthesis, ACC synthase activity is recognized as the regulatory and limiting step. But recently it was found that ACC oxidase transcript levels and enzyme activities also increase in some plant tissues that are induced to form ethylene (Kende 1993; Chen et al. 2005). Thus, ACC oxidase along with ACC synthase regulates the ethylene biosynthesis in plants (Kende 1993; Adams and Yang 1979; Prescott and John 1996; Bleecker and Kende 2000; Chen et al. 2005) (Fig. 17.1).

17.3.2 Ethylene Perception and Signal Transduction

Biochemical and molecular characterization of ethylene biosynthetic pathway has provided insights into the mechanisms by which plants regulate the internal concentrations of this hormone (Kende 1993; Bleecker and Schaller 1996; Sakai et al. 1998). The effectiveness of ethylene as a signal molecule is determined by the capability of cells to scrutinize the varying concentrations of ethylene and transduce this information into physiological responses suitable to the cell type.

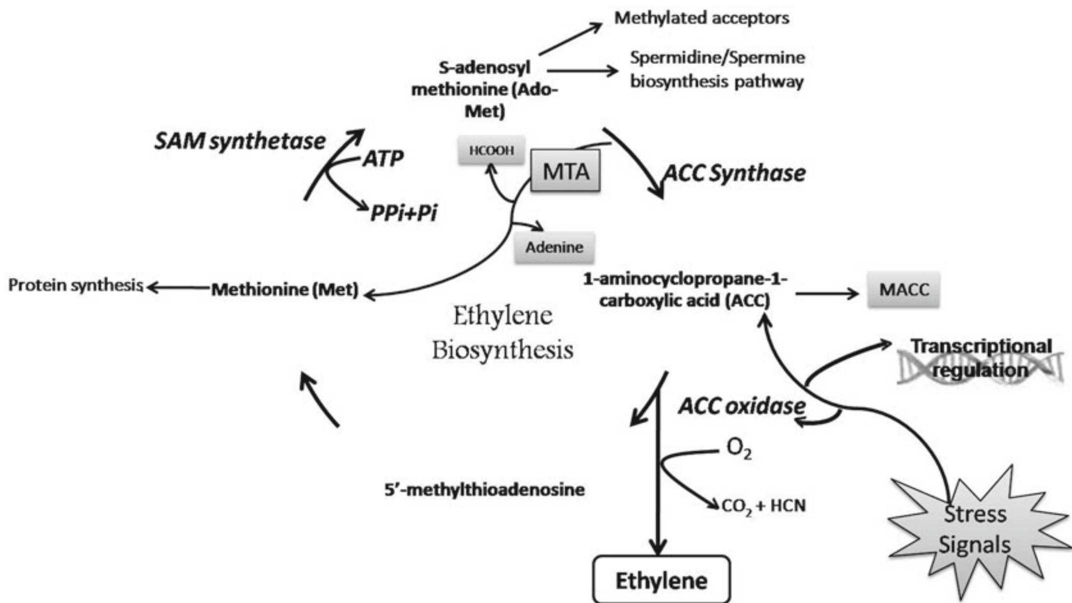

Fig. 17.1 *Biosynthetic pathway and regulation of ethylene*. The formation of *S*-AdoMet is catalyzed by SAM synthetase from the methionine at the expense of one molecule of ATP per molecule of *S*-AdoMet synthesized. *S*-AdoMet is the methyl group donor for many cellular molecules (methylated acceptors), including nucleic acids, proteins, and lipids. In addition, *S*-AdoMet is the precursor of the polyamine synthesis pathway (spermidine/spermine biosynthesis pathway). ACC is the immediate precursor of ethylene. The rate-limiting step of ethylene synthesis is the conversion of *S*-AdoMet to ACC by ACC synthase under most conditions. MTA is the by-product generated along with ACC production by ACC synthase. Recycling of MTA back to methionine conserves the methylthio group and is able to maintain a constant concentration of cellular methionine even when ethylene is rapidly synthesized. Malonylation of ACC to malonyl-ACC (MACC) deprives the ACC pool and reduces the ethylene production. ACC oxidase catalyzes the final step of ethylene synthesis using ACC as substrate and generates carbon dioxide and cyanide. Transcriptional regulation of both ACC synthase and ACC oxidase is indicated by dashed arrows. Reversible phosphorylation of ACC synthase is hypothesized and may be induced by unknown phosphatases (Ptase) and kinases, the latter presumably activated by stresses. Both native and phosphorylated forms (ACC synthase-Pi) of ACC synthase are functional, although the native ACC synthase may be less stable or active in vivo. A hypothetical inhibitor is associated with ACC synthase at the carboxyl end and may be dissociated from the enzyme if it is modified by phosphorylation at the vicinity

Understanding the mechanisms by which plant cells perceive and transduce the ethylene signal has been a difficult problem. Physiochemical considerations have prompted a number of researchers to postulate that ethylene might interact with a receptor through a protein-bound transition metal such as Cu (I) (Burg and Burg 1967; Kovacic et al. 1991; Sisler 1991; Sakai et al. 1998). Detailed studies on a number of mutants of Arabidopsis have revealed that ethylene perception and recognition occurs through a defined pathway (Ecker 1995; Bleecker and Schaller 1996). Recently it has been demonstrated that ethylene is perceived by ER-membrane-bound receptor family. These studies have revealed that ethylene response 1 (ETR1) gene encodes for ethylene receptor in plants and later on the ETR1 protein perceives and transduces the ethylene signal (Bleecker and Schaller 1996; Stepanova and Ecker 2000; Cho and Yoo 2007; Yoo et al. 2009). Genetic epistasis examination of Arabidopsis signaling mutants has revealed that ETR1 is one of the five ethylene receptors along with ETR2 (ethylene receptor 2), ERS1 (ethylene response sensor 1), EIN4 (ethylene insensitive 4), and ERS2 (ethylene response sensor 2) (Bleecker et al. 1988; Chang et al. 1993; Theologis 1998; Bleecker and Kende 2000; Stepanova and Ecker 2000; Schaller and Kieber 2002; Cho and Yoo 2007). These five ethylene membrane receptors found in

Arabidopsis are linked to two-component histidine kinase (HK) sensors found in bacteria and fungi, and the binding to ethylene occurs through *N*-terminal domain localized within the ER (Chang et al. 1993; Theologis 1998; Bleecker and Kende 2000; Binder et al. 2012; Ju and Chang 2012). Two-component systems consist of a sensor molecule with a histidine kinase domain and a response regulator with a receiver domain (Stepanova and Ecker 2000). EIN4, ETR1, and ETR2 consist of both the domains, while ERS1 and ERS2 are devoid of the receiver domain. The absence of the receiver domain indicates that ERS1 and ERS2 either use the receiver domain of EIN4, ETR1, or ETR2 or utilize other response regulators (Hua et al. 1995, 1998; Stepanova and Ecker 2000). The ethylene receptors are largely thought to be redundant because the ethylene receptor is constitutively signaling without ethylene, whereas the ethylene receptor is turned off on addition of ethylene (Qu et al. 2007; Liu et al. 2010). These receptors act as negative regulators through constitutive triple response 1 (CTR1), a genetically identified negative regulator (Kieber et al. 1993; Yoo et al. 2009). Loss of CTR1 function confers constitutive ethylene responses. From genetic and biochemical studies, it has become clear that without ethylene perception, the receptors repress ethylene responses by activating CTR1. Binding of ethylene inactivates ethylene receptor signaling and CTR1 is consequently inactive, thereby leading to ethylene response (Zhong and Chang 2012). CTR1 is presumed to show similarities with Raf, a mitogen-activated protein kinase kinase kinase (MAPKKK) and thus is thought to function like Raf, in a typical MAPK cascade (Kieber et al. 1993; Hahn and Harter 2009; Zhao and Guo 2011; Ju and Chang 2012). The association of CTR1 with the receptor protein complexes occurs chiefly through ETR1 and ERS1 (Kieber et al. 1993; Clark et al. 1998; Huang et al. 2003; Yoo et al. 2009). Moreover a membrane metal transporter EIN2 has an important role in signaling downstream of CTR1 by regulating the accessibility of EIN3 (a key transcription factor) (Chao et al. 1997; Alonso et al. 1999; Guo and Ecker 2003). EIN3 is built up and stabilized in the nucleus to activate hormone inducible primary transcription via ethylene-responsive factor 1 (ERF1) which in turn along with EIN-LIKE1 (EIL1) transcriptional factor initiates the expression of secondary response genes in ethylene-dependent transcription cascades (Alonso and Stepanova 2004; Klee 2004; Gallie and Young 2004; Guo and Ecker 2004; Kendrick and Chang 2008; Yoo et al. 2009). Additionally EIN-LIKE1 (EIL1) and EIN3 are degraded by 26S proteosome in the absence of ethylene (Potuschak et al. 2003; An et al. 2010; Ju and Chang 2012). The protein products of these genes are eventually involved in growth, defense, and survival of the plant by activating ethylene-responsive genes (Yoo et al. 2009; Ju and Chang 2012) (Fig. 17.2).

17.3.3 Nuclear Changes During Ethylene Signaling

Ethylene response results in quick early transcriptional response in the plant via the utilization of protein amendment protocols without de novo production of proteins. Proteomic analysis has suggested that the protein stability control EIN3 (key transcriptional factor in ethylene signaling) is the chief step in ethylene signaling (Chao et al. 1997; Yanagisawa et al. 2003; Gao et al. 2008; Binder et al. 2007). EIN3 accumulation occurs in the nucleus in response to ethylene of 1-amino-1-cyclopropane-1-carboxylic acid (ACC) (Guo and Ecker 2003; Gagne et al. 2004). In the absence of both ACC and ethylene, EIN3 is constantly broken down through 26S proteosome. For this degrading process of EIN3, EBF1 and EBF2 (EIN3-binding F-box proteins) serve as substrates for ubiquitin ligase to degrade EIN3. In ebf1 and ebf2 double mutants, EIN3 protein accumulates and results in strong constitutive ethylene signaling phenotypes (Gagne et al. 2004; Binder et al. 2007). Double mutant experiments with ein3 eil1 have suggested that BF1 and EBF2 act primarily through EIL1 and EIN3 during ethylene signaling. Detailed studies with ebf1 and ebf2 single mutants showed that EBF1 acts during the early stage of ethylene signaling, while EBF2 acts during the later period of ethylene responses (Yoo et al. 2009).

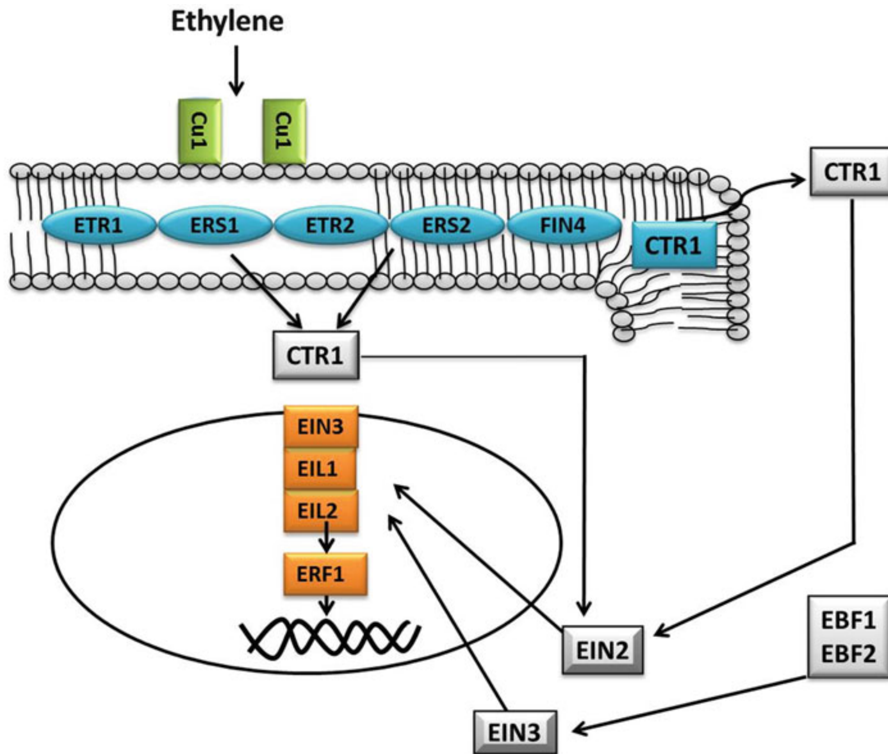

Fig. 17.2 The protein products of these genes are eventually involved in growth, defense, and survival of the plant by activating ethylene-responsive genes

17.3.4 Ethylene Perception at the Endoplasmic Reticulum

Unlike most of the signaling perception mechanisms whose receptors are located either on the plasma membrane or in the nucleus, ethylene signaling receptors are located on ER (Chen et al. 2005, 2007; Grefen et al. 2008; Zhong et al. 2008; Ju and Chang 2012). Various valid justifications have been provided by various workers for localization of ethylene receptors on ER membrane. Firstly, it is a gaseous molecule and can easily diffuse in the cell (Chen et al. 2005; Ju and Chang 2012). Secondly, ethylene receptor genes have originated from chloroplast and evolutionary trends might have resulted in the localization of these genes on ER (Bleecker 1999; Mount and Chang 2002; Chen et al. 2005; Ju and Chang 2012). More convincing argument is that ER is in contact with most of the cell organelles, and thus, the localization of ethylene receptors on ER membrane might facilitate interaction and integration with cellular responses and other signaling pathways (Ju and Chang 2012).

Protein complexes with downstream signaling component CTR1 have been found to act as receptors in ethylene signaling. It has been demonstrated by density gradient centrifugation that CTR1 binds ER membrane via ETR1 as CTR1 cannot bind the membrane by itself as it lacks membrane attachment motifs or transmembrane domains (Clark et al. 1998; Gao et al. 2003; Chen et al. 2005). In addition to ETR1 and CTR1, pull-down assay has confirmed that CTR1 has direct interaction with ERS1 and ETR2 during ethylene signaling. The key role of CTR1 in ethylene signaling and transmission has been proved by various workers (Clark et al.

1998; Cancel and Larsen 2002; Chen et al. 2005). Kinase activity on CTR1 is important in suppressing ethylene response. This was confirmed by using CTR1 mutant which resulted in constitutive ethylene response phenotype. Recent studies have revealed that kinase activity alone cannot confer to suppressing ethylene response, but location of the CTR1 to the endoplasmic reticulum through its alliance with ethylene receptors is also needed (Ju and Chang 2012; Mayerhofer et al. 2012). ctr-8 mutant (a CTR1 mutant), wherein the potential of CTR1 to interact with ethylene receptors has been disrupted, resulted in constitutive ethylene response (Huang et al. 2003; Ju and Chang 2012; Mayerhofer et al. 2012). Same results appear when ethylene receptors are eliminated. The membrane recruitment will place CTR1 with immediate downstream component of the ethylene signaling pathway, i.e., EIN2. EIN2, a subcellular localized protein, contains a highly hydrophobic *N*-terminal domain (Alonso et al. 1999). EIN2 interacts with the ethylene receptors, and its interaction with CTR1 was confirmed by fluorescence resonance energy transfer microscopy using tobacco leaf epidermal cells (Bisson and Groth 2011, 2012). Since EIN2 is located on the endoplasmic reticulum membrane along with the five ethylene receptors and CRT1, interaction between these components may occur simultaneously or synchronously. This interaction syndrome has given rise to "ER-borne ternary super-complex" (Bisson et al. 2009). EIN2 acts at or downstream of CTR1 and either can be involved in protecting EIN2 from proteosome degradation or will promote signaling (Bisson and Groth 2012; Ju and Chang 2012). Depending on the phosphorylated state of the receptor domain, a dynamic interaction might occur between ethylene receptor and EIN2 (Bisson and Groth 2011; Ju and Chang 2012). When histidine was replaced by alanine in ETR1, a fourfold increase in the affinity of EIN2 was seen, while his353Glu substitution had no effect on the interaction (Bisson and Groth 2012; Ju and Chang 2012). In vitro studies have suggested a possible link between ethylene binding and formation of the receptor-EIN2 complex.

17.4 Integration of Ethylene with Other Hormones

Genetic analysis of the plants affected in the "triple response" has led to the wonderful improvement in unraveling and understanding the ethylene signaling processes (Stepanova and Ecker 2000). Recent researches indicate that ethylene affects plant development through signaling networks involving other hormones primarily auxins and gibberellins (Stepanova and Ecker 2000; Yoo et al. 2009).

Arabidopsis mutant gene Auxin resistant1 (AXR1) is characterized by insensitivity to ethylene, but the roots and apical hook of the mutant plant show reduced sensitivity to ethylene also (Lehman et al. 1996; Stepanova and Ecker 2000). Upon cloning, AXR1 showed sequence similarity with E1, a ubiquitin-activating enzyme, and recent studies have shown that AXR1 is involved in ubiquitin-mediated protein degradation (Ruegger et al. 1998; del Pozo et al. 1998). Ethylene-insensitive root1 (eir1) and auxin1 (aux1) auxin mutants show selective resistance to ethylene in the seedling roots (Roman et al. 1995; Pickett et al. 1990). From several types of these mutant experiments, it is clear that ethylene interacts with auxins at various biochemical levels (Stepanova and Ecker 2000).

Under favorable conditions, ethylene regulates various physiological attributes of the plants along with gibberellic acid (GA). The interaction of ethylene with GA has been analyzed using stability of DELLA proteins including GA insensitive (GAI) and Repressor of ga-3 (RGA) in Arabidopsis (Weiss and Ori 2007; Chiwocha et al. 2005; Vandenbussche et al. 2007; Achard et al. 2003, 2007; Yoo et al. 2009). For example, bioactive gibberellic acid levels are low after ACC treatment and in ctr 1 mutant but increase in the ethylene-insensitive etr-2 mutant. DELLA proteins are functionally redundant and negatively regulates all aspects of GA response (Fleet and Sun 2005; Weiss and Ori 2007; Achard et al. 2003, 2006, 2007). Ethylene delays gibberellic acid-stimulated DELLA breakdown and maintains its growth suppressed, while gibberellic acid

elevates plant growth via proteosome-mediated DELLA breakdown (Achard et al. 2003; Fleet and Sun 2005; Weiss and Ori 2007). Expression of bioactive GA synthesis DELLA target genes GA3ox1 and GA20ox1 is stabilized by ctr1. However, the bioactive gibberellic acid levels remain low in ctr1 mutants wherein gibberellic acid is possibly suppressed by a DELLA-independent pathway via regulation by CTR1 or EIN3 (Achard et al. 2007).

17.5 Conclusions

Plants have evolved various different mechanisms to cope up with the changing environment. Plant hormones have played a key role in the regulation of plant growth and development under varying environmental conditions. Ethylene signaling has helped the plants to adapt to these conditions. Ethylene signaling mechanism provided various insights into the role of ethylene in plant development. Much has been elucidated, but puzzles inside in the mechanism of ethylene signaling at various biochemical levels are yet to be unraveled. Genetic, molecular, and biochemical examinations have provided insights into the mechanism of ethylene signaling. With the emergence of molecular genetics, noteworthy advancements towards the explanation of ethylene action in plants have been made during the past few years. Despite our current knowledge about the mechanism of ethylene perception and transduction, many questions remain unanswered. Receptor complexes need to be characterized so as to gain insight into the actual mechanism of ethylene perception and signal transduction. Despite enough confirmation that CTR1 is directly regulated by ethylene receptors, it is yet to ascertain whether it is really so or any other protein motif helps it to bind the ER membrane and get it regulated by ethylene receptors. The function of MAPK cascade in transmission of ethylene signal is still vague and needs to be supported by further molecular evidences. The role of EIN2's association with the ethylene receptors is also intriguing. Further work is required to elucidate and unravel the various receptor complexes which might have a role in ethylene signaling. A detailed investigation of the understanding of the dynamics of protein-protein interactions such as CTR1 and EIN2 complexes is required. The protein involved in ethylene signaling needs to be crystallized for proteomics studies. It will also help in understanding the posttranslational protein modifications as well as the identification of ethylene-responsive targets. These molecular studies will build a dynamic model of the ethylene transduction pathway functional in plants (Ju and Chang 2012).

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Plant, Mycorrhizal Fungi, and Bacterial Network

18

M. Miransari

Abstract

There are some kinds of beneficial symbiotic and nonsymbiotic association between different soil microbes such as arbuscular mycorrhizal (AM) fungi and plant growth-promoting rhizobacteria (PGPR) with their host plants, resulting in the establishment of a sophisticated natural network. The growth of AM fungal spores results in the production of an extensive hyphal network, which can significantly increase the uptake of nutrients and water by the host plant. In the bacterial symbiosis, like rhizobium (as PGPR), the bacteria are able to initiate some cellular structures (nodules), which are actually plant-differentiated tissues and fix the atmospheric nitrogen (N) to be used by the host plant. For the initiation of such kind of symbioses and hence the establishment of the network, signal molecules must be exchanged between the two symbionts. Signal molecules are some kind of biochemical molecules, produced by plant roots and microbes, triggering genetic activation in both symbionts. However, there are some differences differentiating microbial symbiotic association from each other. For instance, AM fungal species are able to colonize a wide range of host plants, with their signal molecules indicating their nonspecific symbiotic association, while rhizobium bacteria are able to establish symbiosis with their specific host plant, which is due to the nature of their signal molecules. It is, therefore, important to indicate the precise details regarding the signal molecules including the plant hormones, which can establish such kind of symbioses and network and the interactions between the microbes. These details can be useful for the production of more efficient inoculums and a more productive and healthy environment. The most recent advancements are presented.

M. Miransari (✉)
Mehrabad Rudehen, Imam Ali Blvd., Mahtab Alley, #55, 3978147395, Tehran, Iran

Abtin Berkeh Limited Co., Imam Blvd., Shariati Blvd. #107, 3973173831, Rudehen, Tehran, Iran
e-mail: miransari1@gmail.com

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Microbes • Plant • Symbioses • Network • Signal molecules

18.1 Introduction

There are different kinds of soil microbes, which are able to establish symbiotic association with their host plant. Arbuscular mycorrhizal (AM) fungi and plant growth-promoting rhizobacteria (PGPR) including rhizobium are among such microbes. AM fungi are able to develop a non-specific symbiotic association with most terrestrial plants. Although in such kind of symbiosis the presence of the host plant is required, the fungal spores are able to germinate in the absence of their host plant. The germination of fungal spores results in the production of a very extensive hyphal network between the soil and plant roots. Such kind of network can significantly increase plant ability to absorb nutrients and water. This can increase the growth of the host plant, especially under stress (Smith and Read 2008).

There are also soil bacteria, which are able to develop a symbiotic (rhizobium) or nonsymbiotic association with their host plant. Rhizobium is among soil bacteria with the ability to develop specific symbiosis with their host plant. This is due to the nature of the signal molecules, produced by plant roots. Such products are able to activate the related bacterial genes, which in response can induce morphological and physiological changes in the plant roots by producing some other signal molecules. There are different kinds of rhizobial strains, which can nodulate their specific host plant. For example, soybean (*Glycine max* L.) is just colonized by *Bradyrhizobium japonicum* (Miransari and Smith 2007, 2008, 2009; Maillet et al. 2011; Singh and Parniske 2012).

Plant growth-promoting rhizobacteria (PGPR) can establish nonsymbiotic association with their host plant. It means that such bacteria reside in the plant rhizosphere and without colonizing plant roots can enhance plant growth by (1) influencing the solubility of soil nutrients; (2) affecting soil properties; (3) producing plant hormones, enzymes, and organic acids; (4) interacting with other soil microbes; (5) controlling pathogens; etc. There are different types of PGPR strains such as *Azospirillum* spp. and *Bacillus* spp. (Jalili et al. 2009; Glick 2010).

Such microbes are important, because they can have profound effects on the environment and plant growth. The production of signal molecules by such microbes results on the establishment of symbiosis with their host plant. There are also interactions between soil microbes, affecting their performance. For example, the presence of soil bacteria on the fungal spores may influence spore germination and their subsequent growth. There are of great environmental and economical significance, for example, in the production of microbial inoculums (Miransari 2011a, b).

There is also another kind of symbiosis, which is called tripartite symbiosis between rhizobium, AM fungi, and their host plant. In such kind of association, the bacteria and the fungi are able to colonize the plant roots at the same time. The bacteria can fix the atmospheric nitrogen (N) for the use of host plant and the fungi can increase the uptake of different nutrients such as phosphorous (P) and water. Such kind of association can be advantageous under different conditions including stress. For example, soybean is able to develop symbiotic association with both *Bradyrhizobium japonicum* and *Glomus* spp. (Mortimer et al. 2012; Miransari et al. 2013b).

There are different products affecting the interactions between plants and microbes, including the phenolic products. Plants and microbes are able to produce such products affecting their symbiotic processes. There are

also kinases, which can affect and regulate the interactions between the host plant and microbes. For example, the protein kinase, Ca^{2+}/calmodulin (CaM) – (CCaMK), is a regulator of rhizobium and mycorrhizal symbiosis and is a receptor for Ca^{2+} signals produced by microbes. The differences in its activity and biding can differentiate between rhizobium and mycorrhizal symbiosis (Shimoda et al. 2012).

18.2 Mycorrhizal Symbiosis

18.2.1 Establishment

In the process of symbiosis between AM fungi and their host plant, the presence of fungal spores in the soil may result in their germination. This can be specially the case when the host plant is present although the spores are able to germinate in the absence of their host plant. The production of some signal molecules by plant roots can trigger the activation of fungal genes and the subsequent growth of spores. There are Myc factors, which are able to activate the symbiotic genes in their host plant roots, similar to the Nod factors in symbiosis process between rhizobium and their host plant (Smith and Read 2008).

Because the association between mycorrhizal fungi and their host plant is not specific, it can have the following implications affecting different plant and microbe interactions. The establishment of common mycorrhizal network may result in the absorption of carbon and nutrients in a plant community by different plant species. This may affect plant and microbial diversity. The symbiont density and composition can affect such a network (Bever et al. 2010).

The most important nutrient, absorbed by mycorrhizal plant, is phosphorous. There are two different pathways for the absorption of P by mycorrhizal plant: the direct and indirect one. In the direct pathway, P is absorbed by P transporters located in plant root hairs and epidermis. However, in the indirect pathway, P is absorbed by the transporters of mycorrhizal hyphae with a several centimeter distance from the root and is translocated to the fungal organelles including arbuscules and vesicles inside the root cortical cells. The induction of P transporters in plant colonized cells results in the transfer of P from the apoplast space to the plant cortical cells (Smith et al. 2011).

When P is deficient, there are different signaling pathways, which are activated in the fungi and in plant to absorb P. In non-mycorrhizal plants the activity of P transporters (PHR1) is enhanced, when P is not sufficient. The transporter is able to bind to the P1BS element in the promoters of P-deficient-related genes and enhances their expression. The increased expression of miR399 genes by PHR1 may result in the translocation of Myc signals, from the root to the shoot under P-deficient conditions in mycorrhizal plants (Smith et al. 2011).

The upregulation of mycorrhizal induced transporter genes, *MtPT4* in the plant root cells with arbuscules, is required at the time of P uptake (Javot et al. 2007). Mycorrhizal plants are also able to absorb significant amounts of N including ammonium and amino acids due to their symbiosis with the fungi (Miransari 2011c). There are several ammonium transporters (AMTs) in plants such as soybean (*GmAMT4*.1), which are expressed in plant cortical cells with arbuscules. Such genes are located on the prearbuscular membranes (branch domains) indicating that their place of activity can be the arbuscule branches (Kobae et al. 2010).

18.2.2 Alleviating Stress

Mycorrhizal fungi are able to increase plant growth under different conditions including stress. Research work has indicated the effectiveness of the fungi under stresses such as salinity, drought, heavy metal, and compaction. The extensive hyphal network is able to signifi-

cantly enhance plant growth by enhancing the uptake of nutrients and water. The fungal hyphae are finer than even the finest root hairs and can grow into the smallest soil micropores and absorb water and nutrients. Interestingly, the fungi can induce some morphological and physiological changes in plant and hence increase plant tolerance under stress. Accordingly, under stress the mycorrhizal plant can alter its physiology in a way so that it can handle the stress (Miransari et al. 2007, 2008, 2009a, b; Miransari 2010).

There are different mechanisms utilized by the fungi to handle the stress. Under salinity stress, the fungi (1) increases plant uptake of water and nutrients; (2) can adjust the rate of Na^+ to K^+, Ca^{2+}, and Mg^{2+}; (3) make the host plant to produce higher amounts of proline; (4) increase plant ability to produce larger amounts of antioxidant products; (5) may also be able to balance the concentration of elements across the cell wall; and (6) allocate saline elements such as Na^+ and Cl^- to the cellular vacuoles and fugal vesicles (Daei et al. 2009; Hammer et al. 2011).

There are also similar mechanisms used by the fungi under drought stress. Increased water uptake can very much help the plant to grow more effectively under stress. The enhanced rate of nutrient uptake can increase plant growth the stress and adjust the concentration of elements in the plant and hence create more favorite water potential. The increased production of plant hormones such as abscisic acid (ABA) can also improve plant behavior under salinity. ABA can control stomatal closure under water stress. This is one of the most important mechanisms by which plant can survive under stress because the amounts of water, which is evaporated from the plant, can very much affect plant growth under stress (Porcel and Ruiz Lozano 2004; Tuteja 2007; Aroca et al. 2008).

In a soil polluted with heavy metals, mycorrhizal plants are able to grow and survive the stress. The fungi can adjust the allocation of heavy metals to different plant parts and tissues including the cellular vacuoles and fungal vesicles. There are also other strategies used by the fungi to alleviate the stress. For example, the production of antioxidants may increase in mycorrhizal plants. The fungi can trigger the activity of stress genes in plant under heavy metal stress. Such kind of activity can result in the production of different compounds such as proteins (methionine), which can absorb heavy metals and allocate them to different cellular parts such as vacuoles. This is important for the adjustment of ion homeostasis and hence plant response to the stress (Miransari 2011d; Rajkumar et al. 2012).

Under compaction, plant growth decreases because the growth of different plant parts, especially the roots, is adversely affected. The cluster growth of plant roots under stress can significantly decrease the uptake of water and nutrients by plant and hence its growth. However, research work has indicated that mycorrhizal fungi are able to alleviate the effect of compaction stress on plant growth by their extensive hyphal network and uptake of water and nutrients (Miransari et al. 2007, 2008, 2009a, b). It has been indicated that relative to the soil microbes under stress, the fungi are able to produce higher amounts of phosphatase and hence make more phosphorous available to the host plant (Taylor et al. 2008; Pupin et al. 2009). This can be very advantageous under the stress and help the plant survive.

18.3 Bacterial Symbiosis

18.3.1 Establishment

There are two different types of association between the soil microbes and the host plant: symbiotic and nonsymbiotic. In a symbiotic association the soil bacteria such as rhizobium are able to reside in the plant roots and induce morphological and physiological changes, which eventually result in formation of nodules. However, in a nonsymbiotic association, the bacteria do not enter the roots and can be interactive with plant roots by the rhizosphere. Rhizobium bacteria are able to specifically colonize the plants from the legume family. Each strain can establish symbiosis with only one plant species, which is related to the nature of the signal molecules, produced by plant roots and the bacteria. As a result

the bacterial genes are activated and produce lipochitooligosaccharides, which in turn can induce morphological changes in plant roots including deformation such as curling and bulging (Wang et al. 2012; Miransari et al. 2013a).

For example, in the symbiosis between soybean and *Bradyrhizobium japonicum*, during the initial stages the host plant is able to produce the signal molecule genistein, which can activate the bacterial genes to proceed with the next stages of symbiosis. After the induction of morphological changes by the bacterial products, the formation of the infection thread can help the bacteria to enter the plant roots and, by affecting plant cellular behavior, result in the production of nodules. It is the place where the bacteria reside and fix the atmospheric N (Zhang and Smith 1995; Long 2001; Miransari et al. 2013b).

The important point about nodulation is the process of autoregulation, which can regulate the number of nodules. For example, in *M. truncatula* the gene *ATB2* can regulate the number of nodules. *MtATB2* can activate a transcription factor and is affected by light and sucrose. The activation of the gene is around the vascular bundle of roots and nodules (Kawaguchi and Minamisawa 2010). The process of N fixation is demanding and hence the plant must use some mechanisms to control the process of nodulation and hence N fixation. The process of autoregulation is by the exchange of signals between plant root and shoot and is controlled by kinases such as CLAVATA1-like receptor. For example, in soybean and *M. truncatula,* the related receptors are NARK and SUNN, respectively (Ferguson et al. 2010).

There are some interesting and important findings regarding the legume plants: (1) the gene sequencing of model legumes have been indicated (Young et al. 2005; Sato et al. 2008); (2) the complete gene sequence of N symbiotic and nonsymbiotic bacteria including *Mesorhizobium loti,* MAFF303099 (Kaneko et al. 2000), *Sinorhizobium meliloti* 1021 (Galibert et al. 2001), *Bradyrhizobium japonicum* USDA110 (Kaneko et al. 2002), and *Azospirillum* sp. B510 (Kaneko et al. 2010) have been shown; and (3) the related genes data base have been established (Kawaguchi and Minamisawa 2010).

18.3.2 Alleviating Stress

Under stress usually the initial stages of N fixation, especially the exchange of the signal molecule between the bacteria and the host plant, is interrupted. Accordingly, the process of symbiosis between the two symbionts and hence N fixation and plant growth is adversely affected. Stresses such as salinity, acidity, and suboptimal root zone temperature may adversely affect the process of N fixation by affecting plant growth (physiology and morphology), as well as bacterial number and activity. However, it has been indicated that the slow-growing bacterium *Bradyrhizobium japonicum* is more tolerant to acidic conditions relative to fast-growing rhizobium, and *Sinorhizobium meliloti* is the most sensitive one (Miransari and Smith 2007).

However, research work has indicated that addition of signal molecule, genistein, may alleviate the stress. Under the stresses of salinity, acidity, and suboptimal root zone temperature, the bacterial inoculums of *Bradyrhizobium japonicum* were preincubated with different concatenations of genistein ranging from 5 to 20 μM under field and greenhouse conditions. Interestingly, genistein was able to partially or completely alleviate the stress. It is believed that preincubation with genistein would activate the bacterial genes under the stress and hence enable the two symbionts to establish the symbiosis. Genistein is also able to increase plant growth by increasing the number and/or the weight of nodules, which are formed faster (Miransari and Smith 2007, 2008, 2009).

Under suboptimal root zone temperature, rhizobium may not be able to detect the presence of the signal molecule, and hence, higher amounts of genistein must be applied to enable the bacteria to establish the symbiosis with the host plant. In a greenhouse research work, Miransari and Smith (2008) stimulated field conditions by using undisturbed soil samples with clay, loam, and sand textures, using aluminum cylinders. At seeding, soybean plants were incubated with *Bradyrhizobium japonicum* treated with different concentration of genistein ranging from control to 20 μM. The pots were then subjected to different temperatures including 14, 19, and 24 °C.

Genistein was able to alleviate the stress by increasing the number and the weight of nodules, the rate of N fixation, and hence plant growth. Interestingly, the effect of genistein was more pronounced in a clay loam indicating the role of soil texture on the process of signal exchange under the stress of suboptimal root zone temperature. The significant interaction effects between genistein and temperature indicated that genistein may be more effective under higher level of stress (14 °C) (Miransari and Smith 2008).

Other research work has also indicated that under higher level of stress, genistein may be more effective (Miransari and Smith 2009). These researchers evaluated the effects of genistein on the process of symbiosis between soybean and *Bradyrhizobium japonicum* under greenhouse saline conditions. Three levels of salinity including control, medium, and high were used to treat the soybean plants inoculated with the bacteria pretreated with genistein connotations at control, 5, 10, and 20 µM. Plants were harvested at three different times (20, 40, and 60 days after seeding). Genistein was able to alleviate the stress and according to the calculations, genistein 11 µM was the most effective concentration under stress.

Genistein can increase the production of Nod factor by rhizobium. The enhancing effects of genistein on the process of symbiosis under stress are because of the following: (1) less signal molecules is produced by the host plant and (2) the sensitivity of bacteria to the signal molecules decreases (Zhang and Smith 1995). Under stress more carbon is alleviated to the roots. Nodules require high amounts of energy for growth and development. Genistein may also intensify the allocation of carbon to the roots and hence provide higher amounts of energy for the use of rhizobium (Miransari and Smith 2009).

18.4 Interactions Between Mycorrhizal Fungi and Bacteria

The soil rhizosphere is a very interesting environment with interactions between plants, microbes, and fungi. The establishment of symbioses between plant and microbes is a function of plant and microbial properties. Plant and microbial growth stage and species can affect the process of symbiosis between the plant and the microbes. The production of plant hormones such as strigolactones, auxin-like products, and volatiles can affect the interactions between the plant and the soil microbes (Bonfante and Anca 2009; Miransari 2012; Miransari et al. 2013a).

Mycorrhizal fungi can be active in association with their host plant and can also interact with the other soil microbes such as PGPR. Such kinds of interactions include the soil bacteria binding to the fungal spores, the production of some molecules by the bacteria into the fungal cells as well as the production of volatiles by bacteria, and the degradation of fungi cellular wall by bacterial products. Such kinds of interactions can affect the fungal performance by influencing the expression of fungal genes and hence its subsequent interactions with the host plant and the other soil microbes (Wamberg et al. 2003; Bonfante and Anca 2009; Miransari 2011a).

It is important to use the appropriate techniques to detect the interactions between microbes with their host plant and with the other microbes, among the most applicable one is the use of proteomics. It is a technique used to study the expression of genes and hence the production of the related proteins and the interactions between proteins during different stages of the symbiotic process. This can be very useful to determine the genes and the related proteins during the symbiotic process. Accordingly, the related molecular pathways can be recognized and modified to increase their efficiency under different conditions including stress. The proteins can be detected using mass spectrophotometry and quantified using the method of stable isotope labeling or label-free method (Jayaraman et al. 2012).

Scheublin et al. (2010) indicated that which bacterial species may interact with fungal hyphae. Using the sequencing of 16S ribosomal RNA (rRNA) and terminal restriction fragment length polymorphism, the bacterial species, which were interactive with the fungal hyphae, were determined. The most interactive bacterial species were from the Oxalobacteraceae family. The presence of

mycorrhizal hyphae in the soil can affect the composition of soil bacteria. This can be related to the interaction of the fungi with its host plant and production of different products by the fungal hyphae (Marschner and Baumann 2003; Rillig et al. 2006; Toljander et al. 2007).

There are different bacterial species, which are able to interact with the fungal hyphae including *Pseudomonas fluorescens, Rhizobium leguminosarum*, *Bacillus cereus*, *Paenibacillus peoriae, and Paenibacillus brasiliensis* (Bianciotto et al. 2001; Toljander et al. 2006). The important parameter regarding the interaction between mycorrhizal fungi and the other soil microbes is the properties of mycorrhizal fungi. Related to the other soil microbes, it is more difficult to investigate the behavior of fungi in the soil due to the following: (1) mycorrhizal fungi are only able to grow in the presence of the host plant and develop the extensive network of hyphae and (2) because the fungal hyphae are very little and not easy to grow and handle. However, research work has indicated that in most of the time the presence and interaction of soil bacteria with the fungal hyphae may enhance the process of symbiosis and hence plant growth (Scheublin et al. 2010; Miransari 2011a).

Scheublin et al. (2010) developed a method to investigate the interactions of mycorrhizal fungi and the other soil microbes. They investigated to determine (1) how the microbes may colonize the hyphal surface, (2) if such kind of colonization is specific, (3) the species of soil microbes, and (4) the changes in the microbial species when interacting with the fungal surface. They grew in vitro cultures of mycorrhizal fungi including *Glomus intraradices* and *G. proliferum* using compartmented plates to make the interactions of fungal hyphae and the isolated soil bacteria likely. Using the microscopy method and the gene sequencing (16S rRNA) of the attached bacteria, the favorite goals were investigated.

There are also some other interesting points regarding the research by Scheublin et al. (2010); they investigated the interactions between the bacteria and the fungal hyphae 20 h after the start of the experiment. It is likely that only the fast colonizing bacteria were detected by the experiment. The effects of hyphal products (sugars and organic acids) on the bacterial species might have also been a very important parameter affecting the diversity of bacterial species attached to the fungal hyphae (Bonfante and Genre 2010).

The fungi and the bacteria are able to feed on each other. The two microbes are able to utilize the products produced by them, and hence, this can affect the nutrient efficiency of the microbes. The ability of bacteria to produce extracellular polysaccharide determines its potential to attach to the plant roots, fungal spore, and hyphae. This may indicate the importance of cell wall composition for the bacterial attachment (Bianciotto et al. 2001). The production of a protein of a lectin type can facilitate the attachment of *Rhizobium* sp. to the fungi (Cerigini et al. 2008).

The interactions between the fungi and the bacteria can benefit the two microbes. Such kind of interaction is especially interesting when the host plant is not present. For example, *Paenibacillus validus* is able to support the growth of *G. intraradices* in the absence of the host plant. It has been indicated that two strains of *P. validus* can enhance the growth of the fungi (up to the level of germinating spores) by producing raffinose and other unknown products (Hildebrandt et al. 2002, 2006). This may indicate that such bacteria are able to produce products similar to the host plant, and their presence may be required for the establishment of symbiosis between the fungi and the host plant (Bouwmeester et al. 2007).

The increased efflux of H^+ during the process of tripartite symbiosis between soybean, mycorrhizal fungi, and rhizobium can affect the process of symbiosis between the symbionts. Both the fungi and rhizobium are able to increase H^+ efflux at the time of symbiosis. The increased efflux by rhizobium is due to the enhanced activity of nitrogenase, in the presence of mycorrhizal fungi. Hence, the acidification of rhizosphere by rhizobium and mycorrhizal fungi can increase the interactions between the symbionts and hence intensify the process of symbiosis (Ding et al. 2012).

18.5 Plant Hormones and Soil Microbes

There are different plant hormones – some of which are produced by soil symbiotic microbes regulating plant and microbial activities. Such hormones include auxin (IAA), abscisic acid (ABA), jasmonic acid (JA), ethylene, salicylic acid (SA), cytokinins, gibberellins, strigolactones, and brassinosteroids (Glick et al. 2007; Jalili et al. 2009; Miransari 2012; Miransari et al. 2013a). There are different molecular pathways activated by plant hormones under stress (Nakamura et al. 2006; Rolland et al. 2006; Truman et al. 2007). Auxin is among the most important plant hormones affecting the development of embryo and fruit, vascular bundle, and root growth. It is synthesized in the stem tip and young leaf and is then translocated to the place of action. There are different soil microbes, which are able to produce auxin (Lejung et al. 2001; Han et al. 2009).

There are different activities controlled by abscisic acid (ABA) in plant including stomatal activity, protein and lipid production, dormancy and development of seed, and tolerance to pathogens. ABA is produced in plant root under different kinds of stress such as salinity and drought and translocated to the plant shoot to control stomatal activity. Accordingly, ABA is the most important plant signal activated during stress (Benschop et al. 2005; Tuteja 2007). In mycorrhizal plants the regulation of ABA is done more efficiently (Aroca et al. 2008).

Ethylene as the stress hormone may be among the most important hormones, which can regulate or be regulated by microbial activities. Under stress the amounts of ethylene increases in plant affecting plant growth and development. However, it has been indicated that PGPR are able to adjust the production and hence activity of ethylene in plant. The bacteria are able to influence the hormone production in plant by affecting the related pathways and hence control the stress. By producing the hormone 1-aminocyclopropane-1-carboxylate (ACC) deaminase, the bacteria can degrade the hormone precursor by the ACC-oxidase pathway (Glick et al. 2007; Yang et al. 2008; Jalili et al. 2009).

Different plant parts are able to produce brassinosteroids including the leaf, shoot, root, vesicular bundle, fruit, flower, and pollen (Tripathi and Tuteja 2007). Such steroidal compounds are usually found with sugars and fatty acids. So far about 70 kinds of brassinosteroids have been found, isolated, and extracted in plant (Sasse 2003; Yu et al. 2008). Such a hormone is able to affect root nodulation and plant growth under non- and stressed conditions by influencing the amount of cytokinin in the nodulated roots (Schilling et al. 1991). Although plant systemic resistance may be affected by brassinosteroids and ABA, the cross talk and interactions between salicylic acid, jasmonic acid, and ethylene include the main signaling pathways affecting plant systemic resistance to pathogens (Miransari 2012).

Jasmonic acid is a lipid plant hormone affecting plant growth and systemic resistance by the related signaling pathways. The enzymes, which are responsible for production of jasmonates, are located in the chloroplast membrane (Schaller and Stintzi 2009). Similar to cytokinin, jasmonates are also able to affect nodule morphogenesis in the legume family. The other plant hormones including ABA, ethylene, auxin, gibberellins, and brassinosteroids are also required for nodule functioning and morphogenesis (Oldroyd et al. 2001). The formation of nodules in *M. truncatula* and *L. japonicus* is also affected by jasmonates (Miransari 2012).

Salicylic acid is also another plant hormone influencing growth and development by affecting plant systemic resistance through the followings mechanisms: (1) expression of priming genes, (2) expression of PAL (phenylalanine ammonia lyase), (3) callose deposition, (4) phytoalexin production, (5) deposition of phenolic compounds, (6) hydroxycinnamoyltyramine deposition, and oxidative burst (Lian et al. 2000; Goellner and Conrath 2008).

There are also strigolactones, as the new classes of plant hormones affecting the process of symbiosis between the host plant and mycorrhizal fungi. The hormone is produced by carotenoid cleavage dioxygenase or 9-*cis* epoxycarotenoid dioxygenase from carotenoids. The hormone is able to affect the host plant growth by affecting

hyphal and shoot branching and the germination of parasitic weeds such as *Striga*. Under P starvation, the production of strigolactones by plant increases (Akiyama et al. 2005; Lopez-Raez et al. 2008; Lopez-Raez and Bouwmeester 2008; Miransari 2011a).

18.6 Conclusion

Plant, mycorrhizal fungi, and bacterial network can be affected by different parameters such as soil, microbes, plant, and climate, under non- and stressed conditions. It is important to evaluate such parameters to increase the efficiency of symbiotic processes and the related established network. The more completed the network, the higher the efficiency of symbiosis. Different molecular and cellular signaling pathways including the hormonal signaling pathways can significantly influence the symbiotic process between the host plant and the soil microbes and hence the related network.

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19 Self-Incompatibility Patterns and Signal Transduction

Latif Ahmad Peer

Abstract

Self-incompatibility (SI), an important mechanism, enables plant species to increase their genetic variability and to avoid inbreeding depression. The recognition and rejection reactions for incompatibility involve a single polymorphic and multi-allelic S-locus. The pistil is enabled by the mechanism of SI to discriminate between self and non-self-pollen. The self-pollen when recognised by the pistil is either rejected just on the stigma or its pollen tube growth is halted in the style. The non-self-pollen is allowed to germinate and grow by the pistil. Plants showing variation with respect to S-locus are termed as haplotypes and are designated as $S_{1,}$ $S_{2,}$ $S_{3,}$ etc. In this review, focus is laid on the discussion about the recent developments in the understanding of three different mechanisms of SI. These mechanisms are controlled by two distinct determinant genes present at the *S*-locus. In *Brassicaceae*, the S-locus encodes two proteins: SRK which is a receptor kinase and a small protein SCR which acts as a ligand. SRK and SCR interact with each other and lead to the incompatible signalling in the papilla cells of the stigma. In case of *Solanaceae*, the rejection response is determined by a female determinant, a ribonuclease, and male determinant, an F-box protein. The mechanism of SI in *Solanaceae* is thought to be accomplished by the degradation of RNA and protein. In case of *Papaveraceae*, only the female determinant is known and the SI mechanism is supposed to involve a Ca^{2+}-dependent signalling cascade which in the end causes the death of non-self-pollen.

Keywords

Self-incompatibility • Signal transduction • *S*-locus • SRK • *S*-locus cysteine rich • *S*-RNase • F-box

L.A. Peer (✉)
Department of Botany, University of Kashmir,
Srinagar 190006, Jammu and Kashmir, India
e-mail: peerlatif@yahoo.co.in

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19.1 Introduction

Self-incompatibility (SI), a well-established mechanism, offers a way to select the mates in plant species in a genetically controlled manner, thereby preventing the mating of those plants that are closely related. In angiosperms, nearly half of the plant species are said to exhibit the mechanism of SI (Kear and McClure 2012). SI response is accomplished by first recognising the self and non-self-pollen grains and then selectively inhibiting the self-pollen or the pollen tube growth of the self-pollen by the pistil. In spite of hard striving efforts, little information is available about the molecular mechanisms which govern these underlying pollen-pistil interactions of self-incompatibility. Pollen-pistil interactions were studied in two genotypes of *Citrus clementine:* a self-incompatible cultivar, 'Comune', and a mutant 'Monreal' that is self-compatible. Profiling the trascriptome of stylar canal cells (SCC) in 'Monreal', indicated that self-incompatibility cessation is accompanied by the differential expression of few specific and mostly uncharacterised transcripts. One such transcript encoded by an F-box gene was found to be drastically upregulated in those stylar canal cells that were micro-dissected by laser and in the whole styles of 'Comune' in which growth of pollen tube was inhibited by self-pollination (Caruso et al. 2012).

It is now proved by the classical studies that a locus, called *S*-locus, multi-allelic in nature, controls the recognition step of SI in majority of the plant species, and if similar '*S*-allele' is specified and expressed by the pollen as well as the pistil, the rejection response happens. The molecules which are found to play their role in the recognition process of SI have been recognised in some families like *Papaveraceae*, *Brassicaceae* (cabbage, *Arabidopsis,* etc.), *Plantaginaceae* (snapdragon), *Solanaceae* (potato, tomato, etc.) and *Rosaceae*. Their identification has only been possible after intense molecular research focused on entities of *S*-locus for the last two decades. The determinant genes identified were found to possess diverse structures, which point towards the fact that SI is represented by collection of divergent systems rather than a single mechanism. From these studies it has now become evident that at least two transcriptional units, linked together and arranged in pairs, are present at *S*-locus. One of these transcriptional units acts as a male determinant while the other functions as female determinant. This *S*-locus multigene complex has been found to show inheritance as a single segregating unit, and the term '*S*-haplotypes' has been assigned to those plants which vary with respect to *S*-locus gene complex.

The two determinants interact with each other at the protein level, and it is at this level the self and non-self-recognition takes place. When both the determinants are from the same haplotype, SI response occurs. In family *Brassicaceae*, both the determinants, male as well as female, have been isolated, and in case of *Rosaceae*, *Solanaceae* and *Scrophulariaceae* male determinants are known; besides it has been found that all these families bear the same type of female determinant. Though the *S*-locus in different SI mechanisms shows a basic scheme of two multi-allelic genes, the determinants which have been identified are different from one another, which suggest that SI has probably evolved several times in diverse lineages of flowering plants.

This chapter aims to highlight the recent work done on self-incompatibility to expound to the reader the present understanding of molecular mechanics of self-incompatibility in flowering plants.

19.2 Sporophytic Self-Incompatibility (SSI) in *Brassicaceae*

Classic studies on self-incompatibility have shown that it is of two types: sporophytic self-incompatibility (SSI) and gametophytic self-incompatibility (GSI). In case of GSI system the haploid genome of the pollen determines its SI phenotype, whereas the diploid genome of the parent from which pollen develops determines SI phenotype of pollen in the SSI system. *Brassicaceae* exhibits SSI system of self-incompatibility. In *B. rapa*, the number of *S*-haplotypes identified are more than 30, whereas for *B. oleracea* the number of *S*-haplotypes

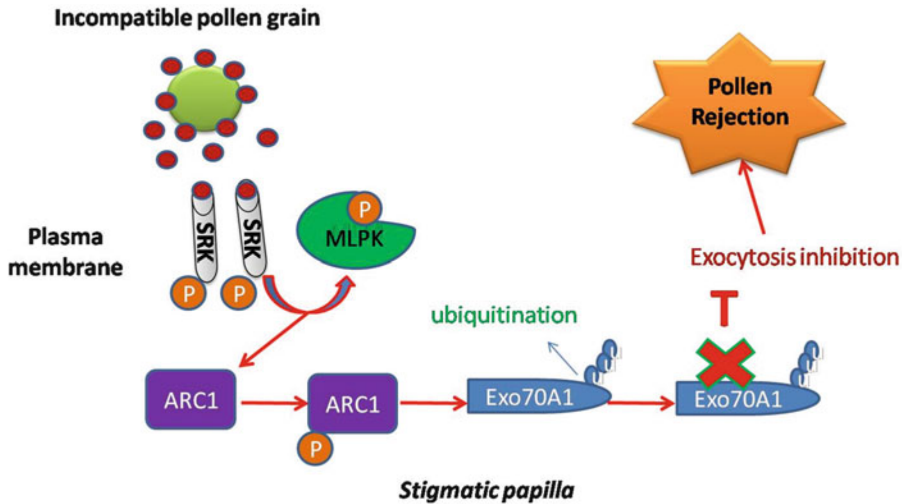

Fig. 19.1 Self-incompatibility signalling in *Brassicaceae*: landing of self-pollen on stigma results SP11/SCR to bind the S-receptor kinase (*SRK*) which in turn activates a signalling pathway in the stigmatic papilla to reject the pollen. Proteins that are downstream in this pathway include the M-locus protein kinase (*MLPK*) and the ARC1 E_3 ubiquitin ligase. When ARC1 gets activated, it inhibits Exo-70A1, a factor needed for pollen hydration and pollen tube growth in stigma

recognised is greater than 50 (Ockendon 2000; Nou et al. 1993). *S*-locus in case of *Brassica* encodes a protein which is a receptor kinase (SRK) and its ligand a small protein (SCR). SRK is expressed in stigma papilla cells, whereas SCR lies within pollen coat. When SRK and SCR share the same allele, the pollen gets rejected. The SI response does not encompass the entire papilla cell but is strictly localised. SRK has been shown to be mostly intracellular and only small amounts of it are found present in particular parts of plasma membrane which acts as the site of interaction with SCR. Once the ligand is recognised, the receptor ligand complex formed is endocytosed and degraded. The self-pollen rejection culminates with the pollen hydration that is abrogated or the growth of the pollen tube is arrested quickly at the stigmatic surface (Fig. 19.1).

19.2.1 Self-Incompatibility Determinants in Family *Brassicaceae*

After lot of molecular research, the female determinant in *Brassica* was found to be SRK, receptor kinase that is expressed in the papilla cells of the stigma and glycoproteins of stigma termed as *S*-locus glycoproteins (SLGs). SLGs represent group of secreted proteins having 50–60 kDa molecular mass, and these proteins have 12 conserved cysteine residues and several *N*-linked oligosaccharides (Takayama et al. 1987). SRK has three domains: extracellular called *S*-domain just like SLG, transmembrane and intracellular domains (Nasrallah et al. 1987).

SLGs as well as SRK are expressed just before the flowers open and match the time at which stigma attains SI. The experiments called gain of function clarified the function of SRK and SLG in the SI (Takasaki et al. 2000). The experiments revealed that when transgenic *B. rapa* was made to express SRK_{28}, it gained specificity for S_{28} haplotype in stigma and S_{28} pollen were rejected; in contrast the transgenic plants in which SLG_{28} was expressed did not display specificity for S_{28} haplotype. Stronger incompatibility against S_{28} pollen was found in the plants which were transformed for both SRK_{28} and SLG_{28} and in such plants only few seeds were produced. So it was demonstrated that specificity for *S*-haplotype of the stigma is determined alone by the SRK, whereas SLG increases the activity of SRK (Silva et al. 2001). *Arabidopsis lyrata* and many genera

of *Brassicaceae* including *Brassica* exhibit a strong SI phenotype even in the absence of SLG expression which supports the view that in the SI response SLG may have different function in diverse *S*-haplotypes (Kusaba et al. 2001).

For male determinant in *Brassicaceae*, pollen coat's small fraction of protein, less than 10 kDa, is accountable for the biological activity that elicits SI response (Stephenson et al. 1997). The successful identification of genes for male determinant named SCR (*S*-locus cysteine rich) or SP11 (*S*-locus protein 11) was made possible by using two approaches, *S*-locus region was cloned and sequenced, and utilising fluorescent-based differential display to hunt for polymorphic gene (Suzuki et al. 1999; Schopfer et al. 1999; Takayama and Isogai 2003). The proteins specified by *SP11/SCR* are small, basic, secreted type and cysteine rich and exhibit high polymorphism with respect to *S*-haplotype. There is a relatively conserved signal sequence which is shared by the alleles but mature proteins specified show high variability, so the protein selection for diversification is strong. Few residues within these proteins show high but not absolute conservation among majority of *S*-haplotypes. These residues include eight cysteines (assigned symbols as C1 to C8), a residue of glycine present between C1 and C2 and a residue of aromatic nature present in between C3 and C4 (Shiba et al. 2002).

The identification of SCR as the male determinant was established by the experiments involving gain of function and pollination bioassay (Shiba et al. 2001; Takayama et al. 2000). In all experiments of the gain of function, it was revealed that the pollen from those plants which was transformed with a particular SP11/SCR transgene developed the specificity with respect to *S*-haplotype of that transgene. The stigma pretreated with self SP11/SCR that was chemically synthesized in the pollination bioassay, exhibited the inhibition of 'cross pollen' hydration as well as penetration. These results thus made it clear that the only male determinant in *Brassicaceae* is SP11/SCR. It was also shown that that within stigma papilla cells, it is SP11/SCR that directly brings incompatible reactions (Kemp and Doughty 2007). It is supposed that tapetal cells secrete SP11/SCR protein in clusters into the locule of the anther where from translocation of these occurs to the surface of pollen (Iwano et al. 2003).

19.2.2 Structure of SP11 Protein

NMR analysis was used to reveal the tertiary structure of S_8-haplotype-specific SP11 protein (Mishima et al. 2003). SI response on S_8 stigma is specifically induced by S_8-SP11 at a minimum dose of 50 fmol on each stigma (Takayama et al. 2001). S_8-SP11 has a structure of α/β sandwich made up of three-stranded β-sheet layer which are twisted and α-helix forming a layer having flanking loops. The structure is stabilised by the presence of four disulphide bonds formed by C1 and C8, C2 and C5, C3 and C6 and C4 and C7. Disulphide bonds and hydrophobic packing mainly stabilise the edges of the loops which is a unique property of SP11/SCR. The α-helix and β_2-strand form the L1 loop. The structure of L1 loop is stabilised by the hydrophobic contact of aromatic residue with the C7 sulphur atom. α-helix is connected to β1-strand by type II β-turn, formed in the nearby segment, that is stabilised by conserved glycine moiety. The L1 loop region of SP11/SCR is suggested to form a domain called hyper-variable (HV) that protrudes from the main protein body, and this domain is essential for *S*-haplotype specificity. The protein surface area contributed by L1 loop region is too little for conferring high affinity offered by the stigma receptor suggesting a requirement for an additional interface (Mishima et al. 2003). The need for additional interface is supported by the results of site-directed mutagenesis (alanine scanning). It is suggested that SRK binding is contributed by the (C3-C4) region that corresponds to L1 loop and the (C5-C6) region that corresponds to L2 loop (Chookajorn et al. 2004). Similarly, the activity of SP11/SCR was abolished when the conserved cysteines or a tyrosine was mutated, whereas the replacement of conserved glycine residue with valine was tolerated. It has been suggested by the experiments of domain swapping that between SCR variants the specificity determinants will be arranged differently.

19.2.3 Mechanism of Transduction of SRK Activation to Inhibit Self-Pollen

There are two signalling molecules that positively mediate the transduction of SRK activation: Armadillo-repeat-containing 1 (ARC1) and *M*-locus protein kinase (MLPK). ARC1 is a protein from stigma that interacts with SRK through its cytoplasmic domain (Gu et al. 1998; Muzzurco et al. 2001). ARC1 is phosphorylated in vitro when the arm repeat region at the *C*-terminal of ARC1 interacts with active kinase domain of SRK. SI response is partially lost when ARC1 expression is suppressed by antisense cDNA so it suggests that ARC1 mediates SI signalling positively (Stone et al. 1999). ARC1 is suggested to contain modified RING-finger and a U-box motif and possess E3 ubiquitin ligase activity that is U-box dependent (Stone et al. 2003). Arm repeat containing 1 is supposed to target and degrades the compatibility factors, resulting in pollen rejection. Though the protein degradation is supposed to be important for successful self-pollen rejection, different proteins whose abundance is altered by SI pathway have remained largely unidentified. When analysis of two-dimensional gel electrophoresis was coupled with matrix-assisted laser desorption ionisation, 56 differential protein spots with 19 unique candidate proteins whose abundance is downregulated following self-incompatible pollinations were identified. The differentials identified are supposed to have a role in different pathways which include signalling, biosynthetic pathways, cytoskeletal organisation and exocytosis (Samuel et al. 2011). The levels of ubiquitinated protein in the pistil were found to increase post-incompatible pollinations, but it was not found true for pistils which were ARC1 antisense suppressed. Besides, SI response is disrupted by proteasome inhibition; therefore, it was proposed that the proteins of stigma responsible for germination of pollen and growth of pollen tube are ubiquitinated and proteasomally degraded when ARC1 is activated by SRK.

Identification of MLPK was made possible when the *modifier* (*m*) gene, a recessive mutant in *B. rapa* var. yellow sarson, was re-examined. The mutant gene leads to self-incompatibility in this plant species (Murase et al. 2004). Earlier the gene was thought to express MIP-MOD, a protein-like aquaporin, but this was later found not the case (Fukai et al. 2001). MLPK, a protein kinase, falls within subfamily of RLCK (receptor-like cytoplasmic kinase) and has monophyletic origin common to receptor-like kinases. The main difference of MLPK from RLCK is that in it transmembrane domain or signal sequences are absent (Shiu and Bleecker 2001). Missense mutation has been found in conserved kinase subdomain VIa of MLPK of variety yellow sarson, and because of this mutation kinase activity of MLPK is lost. A completely compatible phenotype is exhibited by the *mm* plants and the capability of rejecting self-pollen by *mm* cells of papilla can be restored by the transient expression of MLPK. Thus from such results it can be suggested that MLPK positively mediates SI signalling, and also if the pathway of SI signalling is assumed to take multiple routes from SRK, MLPK will be present upstream in this pathway. Furthermore, the MLPK is supposed to function nearby SRK because of the two features: firstly, it has *N*-myristoylation motif that is characteristic of plants at the *N* terminus and, secondly, it exists within plasma membrane of stigma. It is suggested that the SRK signal passed on to MLPK must be returned to SRK; if within SRK signalling, ARC1 is supposed to be the primary component. Such a suggestion is given because ARC1 is one of the downstream effectors of SRK. A signalling complex that may mediate the rejection response is supposed to be formed by MLPK with SRK (Murase et al. 2004).

There are several known components that can regulate the SI signalling pathway negatively. THL1 and THL2 (thioredoxin-h 1 and thioredoxin-h 2), two proteins from the stigma, first recognised in two-hybrid screen of yeast, interact with SRK through a conserved cysteine that is present at its transmembrane domain in a step that does not require phosphorylation (Bower et al. 1996). Under in vitro conditions, THL1 has been shown to prevent SRK auto-phosphorylation when the system lacks the male determinant (Cabrillac et al. 2001). This gives a clue to accept

that THL1 and THL2 negatively regulate the SI signalling pathway by preventing its constitutive activation (Takayama et al. 2001). Another candidate that negatively regulates SI signalling pathway is 'kinase-associated protein phosphatase' (KAPP). Many transformation experiments have proposed that KAPP generally downregulates different receptor kinases in *Arabidopsis*. In stigma cDNA library of *Brassica oleracea,* a homolog for KAPP, SNX1 'sorting nexin', and calmodulins 1 and 2 have been identified (Vanoosthuyse et al. 2003). Like KAPP, SNX1 and calmodulins have also been found to interact with varied types of receptor kinases in vitro. Such interactions suggest that SNX1 and calmodulins may be commonly involved in signalling pathways that are mediated by receptor kinases.

19.2.4 *S*-Locus Evolution in *Arabidopsis* Relatives

Our knowledge of evolution with respect to *S*-locus is limited to the fundamental genes that specify the female determinant 'SRK' and male determinant SCR in *Brassicaceae*. SCR and SRK, the two SI-determining genes, were found to show a pattern of co-evolution that was evidenced by comparing *S*-locus genome organisation of six *S*-locus *Arabidopsis* haplotypes and one haplotype of *Capsella* with *S*-locus organisation of self-compatible haplotype of *Arabidopsis*. By such comparison, *S*-locus genome organisation of common ancestor of *Capsella* and *Arabidopsis* was reconstructed. It was also found that the important factors which contributed in the evolution of these two genes are gene conversion, duplication and positive selection. These factors are supposed to enhance the creation of novel recognition specificities (Guo et al. 2011).

19.3 GSI-Type Self-Incompatibility in *Papaveraceae*

Papaver rhoeas shows GSI system of self-incompatibility wherein pollen's *S*-phenotype is determined by its own *S*-genotype. As many as 66 *S*-haplotypes have been estimated in *P. rhoeas* (Lane and Lawrence 1993). The male determinant has not been identified yet in this system of SI, but an in vitro system of bioassay was developed to study the biochemical events that occur post-self-recognition. In this bioassay, recombinant *S*-proteins were used to inhibit germination of pollen and growth of pollen tube with respect to *S*-haplotype specificity. It has now been revealed by the recent advances that SI controls pollen tube metabolism. During response of SI and due to changes in actin cytoskeleton for longer periods, a soluble pyro-phosphatase is downregulated which leads to programmed death in incompatible pollen tubes (McClure 2007) (Fig. 19.2).

19.3.1 Female Determinant

An in vitro system of bioassay was developed in which self-incompatibility reaction of *Papaver rhoeas* was reproduced and was used to identify the female determinant. The identified female determinant, *S*-proteins, was found to be small secreted-type proteins having molecular mass approximately 15 kDa. Certain *S*-proteins were found to be *N*-glycosylated (Franklin-Tong et al. 2002; McCubbin and Kao 2000). Cloning of about five stigmatic genes, allelic for *Papaver* *S*-proteins, have been done till now. High polymorphism is found in *S*-proteins and these proteins share 51.3–63.7 % common amino acid sequence. The *S*-proteins consists two of four cysteine residues that are conserved and a predicted secondary structure that also is conserved. The secondary structure consists of two α-helices and six β-strands that are joined together by seven surface loops each of which are hydrophilic (Foote et al. 1994). *S*-proteins show variation in amino acid sequence throughout the protein body except for hyper-variable blocks which is different from the *S*-determinants of *Solanaceae* and *Brassicaceae.*

Pollen germination was shown to be inhibited by the *S*-recombinant proteins, produced in *E. coli*, with respect to *S*-haplotype specificity, evidencing for *S*-proteins to be the only female

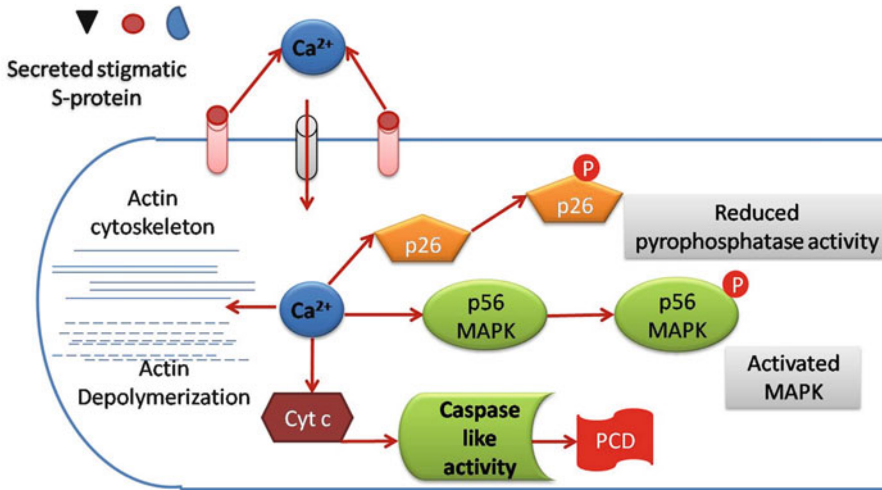

Fig. 19.2 Self-incompatibility signalling in *Papaveraceae*: stigmatic S-proteins interact with putative S-pollen-specific receptor. Pollen grains are rejected in a haplotype-specific interaction which includes rise in intracellular Ca^{2+}-signalling cascade in the pollen. With this, two important events that are phosphorylation of p26 and reorganisation and depolymerisation of F-actin occur within a minute. p56 MAPK is also activated and finally PCD is triggered

determinant. It is also evident from such results that the role of glycan chains in SI recognition as *S*-determinant can be ruled out. Evidences from site-directed mutagenesis favour for some residues in expected loop 6 at surface of the *S*-proteins to be crucial for germination of pollen grains. In S_1-proteins when amino acid, Asp-79, present in loop 6, that shows variability in *S*-proteins of five haplotypes and two nearby conserved amino acids, Asp-77 and Asp-78, were mutated, the S_1-*protein* lost its function with respect to SI response (Kakeda et al. 1998).

19.3.2 Male Determinant

Challenging *S*-proteins on pollen tubes resulted in an immediate increase in the concentration of $[Ca^{2+}]_i$ (intracellular free calcium) in shank region of pollen tube, about 50 μm behind the tip of the pollen tube. The rapid increase in $[Ca^{2+}]_I$ happens because ofCa^{2+} influx, so a supposition is made that the male determinant may be a receptor present in the membrane, linked with Ca^{2+}-transporting channels (Franklin-Tong and Franklin 2003). Biochemical studies pointed towards a pollen-specific membrane receptor, '*S*-protein-binding protein' (SBP), that specifically interacts with stigmatic *S*-proteins but not with respect to *S*-haplotype specificity. SBP is therefore suggested to be the only the accessory receptor and not the male determinant (Hearn et al. 1996).

19.3.3 Signalling Cascade in the Incompatible Pollen of *Papaveraceae*

In incompatible pollen of *Papaver rhoeas,* the growth of the pollen tube is inhibited, cytoskeleton is altered and finally programmed cell death is initiated by the signalling network that is dependent on Ca^{2+}. This signalling network is induced by the interaction of cognate male and female *S*-determinants, Prps, transmembrane protein expressed in pollen, and PrSS, secreted-type protein expressed in pistil (de Graaf et al. 2012). A rapid rise of $[Ca^{2+}]_I$ within the pollen tubes is somehow induced by the *S*-haplotypic interaction between supposed pollen receptor and *S*-protein of stigma. Challenging pollen tubes by *S*-proteins that are incompatible, pollen tubes show increases of $[Ca^{2+}]_i$ in their shank region within a few seconds and continues for several minutes (Franklin-Tong and Franklin 2003).

With the increase in $[Ca^{2+}]_i$ in the pollen tube's shank region, the pollen tube tip shows a quick decrease in high gradient of oscillating $[Ca^{2+}]_i$. $[Ca^{2+}]_I$ is proposed to act as a second messenger that induces number of signalling cascades intracellularly. These cascades culminate with the immediate tube growth inhibition and incompatible pollen death. So it has been interesting to unravel the signalling components that work downstream the early signals of Ca^{2+}. All cells that show tip growth such as neurons and pollen tubes exhibit a typical high oscillating Ca^{2+} at their tips. The biological significance of $[Ca^{2+}]_i$ gradient is unclear but its loss is expected to have role in the pollen tube growth inhibition. An immediate physiological change that occurs in SI response is that the pollen tube actin cytoskeleton is dynamically rearranged. This rearrangement includes loss of several F-actin bundles and formation of punctate foci by these lost actin bundles. Such rearrangement occurs within 1–2 min of post-challenge by an incompatible *S*-protein (Geitmann et al. 2000). SI also causes cortical microtubule depolymerisation and this depolymerisation unlike actin cannot undergo reorganisation (Poulter et al. 2008). Two different proteins that bind to the actin, profilin and gelsolin, are supposed to be part of the Ca^{2+} signalling that results in depolymerisation of actin (Huang et al. 2004).

p26, a cytosolic protein from pollen having molecular mass of 26-kDa, is another early target of the SI response. p26 undergoes initial phosphorylation within 90 s post-challenging of pollen by incompatible *S*-protein, and a large increase has been reported during the following 400 s. This phosphorylation step is also expected to be due to SI signalling that is mediated by Ca^{2+} which is supported by observation that the time of increase in phosphorylation of p26 matches to that of the $[Ca^{2+}]_i$ increase and the protein kinase that phosphorylates p26 depends on calmodulin and Ca^{2+} (Franklin-Tong and Franklin 2003). As revealed by sequence analyses, p26 shows 80 % similarity in the amino acid sequence within organic pyro-phosphatases of plants, and its activity was proven by biochemical assays on recombinant protein. In addition, phosphorylation reduces the pyro-phosphatase activity of p26 which indicates that activity of p26 is probably affected due to SI response (Franklin-Tong and Franklin 2003). It is also suggested that p26 inactivation due to SI response may result in reduction of biopolymers which are required for the growth of pollen tube tip hence inhibiting the growth of pollen tube.

Another target of SI signalling is p56 which is a MAPK (putative mitogen-activated protein kinase). Its being MAPK is evidenced by the fact that it can react specifically to TEY antibody and loses kinase activity in presence of apigenin. p56 responds late to the SI signalling, and its activation is supposed to be downstream of SI signalling initiated by Ca^{2+}. Its being downstream in the signalling cascade is evidenced as when lanthanum, which blocks the calcium channels, is treated on growing pollen, it gets inactivated (Rudd et al. 2003).

19.3.4 Triggering of PCD by SI Response

Recent studies of SI on *Papaver rhoeas* suggest that the activation of MAPK has a part in the initiation of programmed cell death in the cells. PCD of incompatible pollen tubes is supposed to be induced by the challenge of SI (Thomas and Franklin-Tong 2004). The fragmentation of nuclear DNA characteristic of PCD was seen to start after 4 h of challenging pollen tubes by the *S*-proteins that are incompatible and increased up to 80 % within 14 h. This DNA fragmentation is inhibited when the pollen tubes are treated with a tetra-peptide DEVD that inhibits caspase-3. To date no homologue of caspase is known in plants but a caspase-like activity is suggested in SI signalling. MAPK's role in SI-controlled viability and cell death of pollen is evidenced by the fact that U0126 'rescues' incompatible pollen, while U0124, a negative analogue of U0126, cannot. It should be noted that U0126 prevents p56 activation that is induced by SI in incompatible pollen. Both DEVDase activity and DNA fragmentation are reduced considerably in the presence of U0126 that implies MAPK's involvement during early events of PCD (Li et al. 2007).

Leakage of cytochrome c from mitochondria also induces SI response in incompatible pollen tubes. The leakage occurred just after 10 min of SI induction and showed a gradual increase till 120 min. The caspase-like activity has been implicated in the signalling of SI which was reinforced by the analysis of activity of the enzyme involved in cleavage of PARP (poly (ADP-ribose) polymerase). The incompatible pollen tubes showed PARP cleavage activity just after 2 h of SI induction and this cleavage activity increased over time. An artificial increase of $[Ca^{2+}]_I$ in pollen tubes sequentially induces the hallmarks, i.e. fragmentation of nuclear DNA, leakage of cytochrome c and cleavage activity of PARP, of programmed cell death, which suggests that all of these are part of signalling cascade of SI that is mediated by Ca^{2+}.

19.4 *Solanaceae* Type: *S*-RNase-Based Self-Incompatibility

Rosaceae, *Scrophulariaceae* and *Plantaginaceae* exhibit the same type of self-incompatibility as *Solanaceae.* In this type of self-incompatibility, there are two genes, *S*-RNase and SLF/SBP at *S*-locus, both of which are polymorphic. These genes control the specificity of self/non-self-pollen recognition. The female *S*-determinant is an *S*-RNase and is alike in *Scrophulariaceae*, *Solanaceae* and *Rosaceae* (Kao and Tsukamoto 2004). As *S*-RNase was first recognised in family *Solanaceae*, the type of SI which is mediated by *S*-RNase has been named after *Solanaceae*. In this gametophytically controlled (GSI) system of SI, there ejection of self-pollen occurs in style during the course of growth of the pollen tube (Fig. 19.3).

19.4.1 *S*-RNAse as the Pistil Specificity Determinant

It was Anderson et al. (1986) who identified the *S*-RNase gene in *Nicotiana alata*. The *S*-RNases are style glycoproteins having molecular mass approximately 30 kDa. Style proteins have been shown to have same catalytic domain asT-2 type ribonucleases of fungi. These proteins have been termed as '*S*-RNases' because of having ribonuclease activity (McClure et al. 1989). *S*-RNases are pistil-specific proteins, produced in the transmitting cells and sent to transmitting tract where it is localised in extracellular space. The upper 3rd part of style is the site where *S*-RNase is most abundant, and this location matches the site where incompatible pollination leads to the self-pollen tube growth inhibition (Ai et al. 1990). Immature pistils are unable to display SI and show low levels of *S*-RNase, whereas mature pistils show high levels of *S*-RNase which accounts for up to 10 % of total protein from pistil (Roalson and McCubbin 2003). Experiments pertaining to gain of function verified the role of *S*-RNases in SI signalling (Murfett et al. 1994; Lee et al. 1994). One or more glycan chains are linked to *S*-RNases. In *Petunia inflata* the S_3-RNase was engineered by removing the *N*-glycosylation site, and it was shown that it continued to show its capability of rejecting S_3-pollen. This result favour protein backbone and not the glycan chains of *S*-RNases to the site where *S*-haplotype specificity determinant capability resides.

The *S*-RNase shows high divergence, and in species belonging to *Solanaceae* the sequence of amino acid identity has been found to be between 38 and 98 % (McCubbin et al. 2000b). Notwithstanding the high sequence diversity, there are several conserved regions present in *S*-RNases-like five conserved regions in solanaceous *S*-RNases symbolised as C1, C2, C3, C4 and C5. Rosaceous and scrophulariaceous *S*-RNases are structurally similar to solanaceous ones except for C4 region being absent. C2 and C3 regions contain a conserved catalytic histidine residue and are identical to T2 RNase from fungi. Solanaceous *S*-RNases has two hyper-variable regions which are termed as HVa and HVb, whereas in rosaceous *S*-RNases single hyper-variable region, which correspond to HVa of Sf11-RNase from *Solanaceae*, is present. Crystal structures of Sf11-RNase and S_3-RNase from *Nicotiana alata* and *Pyrus pyrifolia,* respectively, are now known (Ida et al. 2001). Both of these *S*-RNases are structurally identical, containing

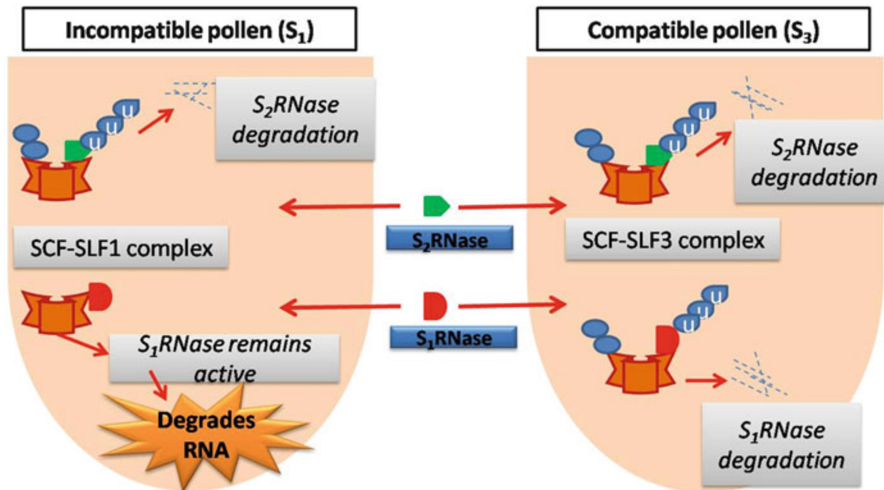

Fig. 19.3 Self-incompatibility signalling in *Solanaceae*: upon compatible pollination, SLF proteins forms active SCF complex which ubiquitinates and degrades *S*-RNase. In incompatible pollinations, SLF protein fails to form a functional SCF complex to inactivate *S*-RNase resulting in its degrading RNA and inhibiting growth of pollen tube

seven β-strands and eight helices. The HV regions of the two *S*-RNases exhibit the notable difference. The hyper-variable regions HVa and HVb of Sf11-RNase consists of a long loop that is positively charged, a part of α-helix and a short α-helix that is negatively charged. These HV regions are expected to form determinant specific domain because they are placed near one another and exposed to the surface. This model is supported by experiments of domain swapping (Lai et al. 2002; Matton et al. 1997). The two *S*-RNases, S11-RNase and S13-RNase, of *S. chacoense* vary only in ten amino acids, and three of them are present in the HVa, while one is present in HVb region. Transgenic plants of *S. chacoense* expressing hybrid *S*-RNase made by substituting amino acids in hyper-variable regions of S11-RNase by those of S13-RNase showed rejection response towards pollen of *S*13 haplotype but not *S*11 haplotype. Thus suggestions are given in favour of hyper-variable regions to be actively involved in determination of specificity with respect to *S*-haplotype, but other regions may also be involved. In case of rosaceous *S3*-RNase, the HV region is identical to that of Solanaceous Sf11-RNase except for the α-helix that corresponds to HVb of Sf11-RNase is not present in Rosaceous *S*3-RNase (Matsuura et al. 2001). It is thus suggested that in the rosaceous *S*-RNase, the interacting domain is formed by the HVa region alone.

The *S*-RNases are cytotoxins which specifically prevent the incompatible pollen tube growth. They are also thought to act as recognition proteins (Matton et al. 1999; McClure et al. 2011). Although *S*-RNase regulates *S*-haplotype specificity alone, it is suggested that other factors of style like HT-B are required to confer full functionality to *S*-RNase (Cruz-Garcia et al. 2003). HT-B, small protein rich in asparagine, was first found in the *N. alata,* a self-incompatible species, while *N. plumbaginifolia,* a self-compatible species, lack HT-B (McClure et al. 1999). Later homologues of HT-B were found in *Solanum* and *Lycopersicon* (Kondo et al. 2002; O'Brien et al. 2002).

19.4.2 F-box Protein: The Male Determinant in Family Solanaceae

It proved harder to identify the gene encoding the pollen specificity determinant than the female determinant. Not any potential candidate for pollen determinant was found from the attempts

which were used to identify proteins from the pollen or pollen tubes that depicted differences with respect to haplotype specificity in isoelectric point and molecular mass. The efforts to identify proteins from pollen or pollen tube that show interaction with *S*-RNase were also unsuccessful in identifying male determinant (Dowd et al. 2000). Again RNA differential display of *S*-locus used to detect genes specific for pollen also failed to give any clue of male determinant, because all genes which were identified depicted minimum degree of diversity with respect to allelic sequence at the amino acid level. So none of these genes was deemed fit as *S*-locus candidates (McCubbin et al. 2000a; Wang et al. 2003). F-box proteins encoding genes were isolated by use of RNA differential display and these later came out to be SLF-like proteins (Hua et al. 2008).

A breakthrough for male determinant came when sequence of *S*-locus was studied in a member from family *Scrophulariaceae*, *Antirrhinum hispanicum.* It was found that S_2-haplotype has new F-box gene which was termed AhSLF-S_2-haplotype. In S_2-haplotype, gene expression of AhSLF is restricted to anther and pollen (Lai et al. 2002). However, other *S*-haplotypes do not possess any gene allelic to AhSLF-S_2, whereas counterpart genes in other *S*-haplotypes show exceptionally high similarity in sequence with it. So the question still remains there whether AhSLF-S_2 specifies a pollen *S*-determinant. Four F-box genes were recognised in the genomic region of *S*-locus ~60-kb nearby gene encoding *S*-RNase in *Prunusmuma,* a member of *Rosaceae*, by genomic analysis of its *S*-locus (Entani et al. 2003). But only one of these genes, named *PmSLF*, exhibited the characteristics of a male determinant. These characteristics include its location in the *S*-locus genomic region that is highly divergent, exhibiting diversity with respect to *S*-haplotype specificity and its expression explicitly in pollen (Entani et al. 2003). *Prunus dulcis*, *P. cerasus* and *P. avium* also have *S*-locus F-box genes that are polymorphic and are individually named SFB (*S*-haplotype-specific F-box) (Yamane et al. 2003). *Prunus* SLF/SFB satisfied all characteristics to be a pollen *S*-determinant. It consists of two hyper-variable regions named HVa and HVb, at the *C* terminus (Ushijima et al. 2003). In apple (*Malus domestica*), two analogous F-box genes were found by sequencing317 kb region of S-locus of apple *S9-haplotype*. In *Pyrus pyrifolia* and apple, different haplotypes exhibit polymorphic genes homologous to SLF. There are two or three similar genes at *S*-locus of each *S*-haplotype so the genes have been called SFBB (*S*-locus F-box brothers). The SFBB regions which are variable are positively selected and expression of these genes occurs explicitly in pollen (Sassa et al. 2007). It was made clear by the phylogenetic analysis of sequences of *S*-RNase and SFB that relationships exhibited by haplotypes of *Prunus cerasus* L. have not been found in any taxon within *Prunus.* Thus evolution of these genes has accompanied with the preservation of polymorphic regions at *S*-locus (Tsukamoto et al. 2005).

The transformation experiments in *Petunia inflate* finally gave the substantial evidence in favour of SLF/SFB for being the male determinant (Sijacic et al. 2004). In *Petunia inflata, PiSLF* (polymorphic F-box gene) was identified near *S-RNase* during search for male determinant in a large region of *S*-locus. A well-known phenomenon termed 'competitive interaction' was utilised to determine whether *PiSLF* is a male *S*-determinant. Tetraploid plants often exhibit the competitive interaction and the hetero-allelic diploid pollen grains (possessing different haplotypes) formed and fail to act in SI, whereas homo-allelic diploid pollen grains (possessing same *S*-haplotypes) act in SI. The mechanisms working at molecular level for breaking down of SI in hetero-allelic pollen is unknown (de Nettancourt 2001). Consistent with this phenomenon, the pollen function broke down in SI of *S*1*S*1, *S*1*S*2 and *S*2*S*3 plants when they were transformed with *PiSLF*2 (*PiSLF S*2-allele). Also, when *S*1*S2/PiSLF*2 and *S*2*S*3/*PiSLF*2 progenies after self-pollinations were genotypically analysed, it showed that self-compatibility is exhibited by the *S*1 and *S*3 transgenic pollen having *PiSLF*2 as transgene, but not by *S*2 transgenic pollen. So the long hunted male determinant was confirmed to be the SLF/SFB.

19.4.3 Mechanism of *S*-Haplotype-Specific Pollen Inhibition

Even though both the determinants, female and male, are known, the molecular mechanisms underlying the interaction of these molecules leading inhibition of incompatible self-pollen growth are not clear. Knowing that RNases have ribonuclease activity and these enter into the pollen tubes irrespective of whether these tubes are compatible or incompatible, it is supposed that *S*-RNases destroy the RNA of self-incompatible pollen and hence act as cytotoxins. SLF/SFB has an F-box motif which enables it to interact with proteins from E3 ubiquitin ligase enzyme complex (Gagne et al. 2002). It should be noted that this enzyme ligase complex along with E2 enzymes ubiquitinate proteins that are to be targeted, and later in several cases these ubiquitinated target proteins are acted upon by 26S proteasome and degraded.

19.4.3.1 Self-Pollen Rejection at Biochemical Level

The presence of self as well as non-self-pollen *S*-RNases in the same pollen tube makes it clear that *S*-RNases enter into the pollen tubes irrespective of haplotype specificity (Luu et al. 2000). So any model favouring selective uptake of *S*-RNases by the pollen tubes in SI is rejected (Kao and McCubbin 1996). In the initial steps, *S*-RNases are sequestered in vacuole-like compartments, and in incompatible tubes these compartments later get disrupted thus releasing *S*-RNases into the cytoplasm (Goldraij et al. 2006). For successful SI response, it is necessary that the *S*-RNases' hold finally reach the cytoplasm where it has to act as a cytotoxin (Huang et al. 1994). The site of action of *S*-RNase was supported by the observation of compartmentalisation of *S*-RNases that were expressed ectopically in both self and non-self-tubes. Further the viability of pollen and SI response was not affected when *S*-RNase was directly expressed in pollen. This has also been found true for localisation of SLF (Hua et al. 2007; Meng et al. 2009). After entering the pollen tube, only self *S*-RNase can exert its cytotoxic activity and not the non-self-one. Studying PiSLF at the biochemical level has paved way for studying this observation and unravelling its molecular mechanism. The protein degradation involves activation of ubiquitin by enzyme E1, conjugation and ligation of ubiquitin chains to target proteins by E2 and E3, respectively, and then transfer of target proteins by them to 26S proteasome. One of E3 ubiquitin ligase enzyme complex, SKP1-CULLIN-F-box (SCF) consist F-box protein as their part (Smalle and Vierstra 2004). In SCF the F-box protein assists in the specific degradation of specific proteins by identifying and interacting with them. Because of the presence of F-box domain in SLF, it may be hypothesised to be the part of SCF that can be utilised to recognise non-self and incompatible *S*-RNases within the pollen tubes and mediating their specific degradation. This also makes clear how only self *S*-RNases can function within pollen tubes.

There are four proteins, F-box protein, RBX1 (RING-HC finger protein), Skp1 and Cullin, in SCF complex (Moon et al. 2004). Cullin interacts with Rbx1 and Skp1 through the *C*- and *N*-terminal domains, respectively. Skp1 interacts with F-box domain of F-box and F-box protein contains many other domains containing leucine-rich repeats, WD40-repeats, etc. which interact specifically with protein substrates (Cenciarelli et al. 1999). But SLF lacks any protein interacting domain at its *C*-terminal. *Antirrhinum* has SLF-containing SCF complex consisting also SLF-interacting Skp1-like protein (Ssk1) and new Skp1-like protein (Zhao et al. 2010). Expression of Ssk1 is specific to pollen but the gene encoding is monomorphic. Ssk1 displays characteristic amino acid tail that forms a disordered coil downstream *C*-terminal "WAFE" residues (Lee et al. 2008). The characteristic tail of Ssk1 is expected to interact with AhSLF2. In *P. inflata*, E3-ubiquitin ligase complex is supposed to contain PiSLF, Pi SBP1 and Pi-CUL1-G, but not Rbx1 or Skp1 (Risseeuw et al. 2003). In this complex, PiSBP1 is supposed to act as a bridge between PiSLF and PiCUL1-G and interacts with E2-like Rbx1. PiSBP1 has a size that is three times that of PiRBX1; this supports for its dual role (Hua and Kao 2006).

19.4.3.2 Primoideae, Subfamily of *Rosaceae* Shows Deviation from General *S*-RNase Governing SI Mechanism

Now it became clear that the mechanisms employed for SI recognition in *Rosaceae*, *Plantaginaceae* as well as *Solanaceae* involve the male and female determinants as SLF/SFB and *S*-RNase, respectively, but the interaction between these two determinants in these families may not show complete conservation (Huang et al. 2008). Primoideae, subfamily of *Rosaceae*, does not display phenomenon of competitive interaction in contrast to its presence in *Maloideae*, *Plantaginaceae* and *Solanaceae* (Adachi et al. 2009; Golz et al. 2001; Hauck et al. 2006; Xue et al. 2009). In Primoideae, mutations in SLF result in SI breakdown which is in contrast to the SLF mutations in Plantaginaceae and Solanaceae that usually result in pollen rejection response by pistils irrespective of *S*-genotype but such mutants do not occur in self-compatible lines (Ushijima et al. 2004; Yamane and Tao 2009). Thus SLF in Primoideae is supposed to protect specifically self as well as non-self *S*-RNases from degradation in contrast to its role in degradation of non-self *S*-RNases in *Maloideae*, *Plantaginaceae* and *Solanaceae*. Primoideae also differ significantly from Maloideae, *Plantaginaceae* and *Solanaceae* with respect to SLF copy number (Sassa et al. 2010). The subfamily Primoideae also differs in *S*-locus configuration from the Maloideae. The *S*-locus of Maloideae consists of two F-box genes, SFBBs encoding male determinants, and is homologous to those in apple. Additionally 20 more SFBB-related genes have been recognised in Maloideae suggesting larger size of *S*-locus than *Primoideae* in it (Minamikawa et al. 2010). *Plantaginaceae* exhibits three AhSLF-related genes showing high similarity in sequences of amino acids with Ah SLF-S2 (Zhou et al. 2003). In contrast additional F-box genes have been found in the *S*-locus of *Primoideae,* but these genes exhibit lower diversity with respect to allelic sequence of SLF thus are not believed to be involved in SI.

19.5 Conclusion

It is now evident that self-incompatibility is regulated by multigene complex, *S*-locus, consisting a minimum of two linked genes always in pairs, one encoding female determinant and the other male determinant. The genes of this locus segregate together and are inherited as single segregating unit, and the plants varying with respect to this gene complex are called *S*-haplotypes. The interaction of proteins of two determinants from same haplotype results in SI response.

Though the *S*-loci have structural commonalities, the determinants encoded in *Solanaceae*, *Brassicaceae* and *Papaveraceae* are different and completely different self/non-self-recognition mechanisms are employed in these families. SI has been said to have two different types, gametophytic self-incompatibility and sporophytic self-incompatibility, on the basis of genetic studies. At molecular level, GSI exhibits two different mechanisms, *Solanaceae* and *Papaveraceae* types. In SSI only one mechanism, *Brassicaceae* type, has been expounded at molecular level, but it has been suggested that different mechanisms may be present.

The dissection of *S*-locus in *Brassicaceae* revealed presence of SP11, SRK and SLG genes. SP11 encodes male determinant specifically in the tapetum which later accumulates in the pollen coat. SRK encodes female determinant that is localised in stigma papilla membrane. SLG localises cell wall of papilla which is not important for SI but only increases SI response of some haplotypes. When self-pollen reach the stigma, SP11 enters the cell wall of papilla and interacts with SRK with respect to haplotype specificity. This specific interaction leads auto-phosphorylation of SRK thereby initiating a signalling cascade that finally ends with the rejection of self-pollen. This signalling cascade has not yet been elucidated but positive effectors of it like ARC1 and MLPK have been recognised. It has been shown that SCR and SRK have co-evolved in *Capsella* and *Arabidopsis;* their evolution has been profoundly affected by gene conversions, duplications, and positive selection.

In case of *Papaveraceae*, the female determinant protein, encoded exclusively in stigma, is *S*-protein. It interacts with putative male determinant with respect to haplotype specificity and this interaction causes influx of Ca^{2+} into the shank region of pollen tube. SPB is supposed to be involved in such influx. Ca^{2+} influx leading to $[Ca^{2+}]_i$ increase initiates a signalling cascade which ultimately leads to tube growth inhibition and programmed cell death of self-pollen. The downstream cascade includes the actin cytoskeleton rearrangement and depolymerisation of cortical microtubules. $[Ca^{2+}]_i$ also results in inactivation of p26 and activation of p56. Inactivation of p26 is proposed to lead to the tube growth inhibition by reducing biopolymers vital for growth of the tip. Lastly SI response leads to the induction of PCD in incompatible pollen tubes.

Solanaceae-type self-incompatibility, the mechanism of which is common in *Rosaceae*, *Plantaginaceae* and *Scrophulariaceae*, is regulated by two polymorphic *S*-locus genes, SLF and *S*-RNase. The *S*-RNase is the female determinant. When the self-pollen tubes grow through the style, *S*-RNase enters them and destroys the RNA. Entry of *S*-RNase into the tubes is irrespective of haplotype specificity but RNA is destroyed only in self-pollen tubes. Because of such specific degradation of RNA, *S*-RNases are believed to have role in recognition and functioning as *S*-specific cytotoxins. SLF, an F-box protein, functions as male determinant. It is a part of E_3-ubiquitin ligase complex and is hypothesised to initiate degradation of non-self *S*-RNases specifically within the pollen tube. In subfamily *Primoideae* of *Rosaceae*, SLF is supposed to specifically prevent the degradation of self as well as all non-self *S*-RNases.

Current studies support the view that SI evolution has been independent and occurred several times in many lineages of flowering plants. It is also evident that a large number of gene families have contributed to the determinant genes including even those members whose expression occurs in vegetative tissues (Vanoosthuyse et al. 2003). With the further developments in understanding of SI, it would be interesting to determine the similarities and differences in divergent mechanisms which are involved in SI system of other families of angiosperms.

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Plant Disease Resistance Genes: From Perception to Signal Transduction

20

B.A. Padder

Abstract

Plants defend themselves against pathogen attack by activating a multicomponent defence response. The pathogen invasion is recognised by proteins encoded by plant disease resistance (*R*) genes that bind specific pathogen-derived avirulence (*Avr*) proteins either directly or indirectly via guard to decoy to mechanistic model. As a result, an intracellular signal transduction cascade is initiated, triggering activation of the defence arsenal of the challenged host plant cell and resulting in a localised cell and tissue death at the site of infection and a non-specific systemic acquired resistance (SAR) throughout the plant, which prevents the further spread of the infection termed as hypersensitive response (HR). A large number of plant resistance genes have been grouped into eight basic classes that can be grouped into several superfamilies, based on their protein domains. The vast majority of genes cloned so far belong to the NB–LRR, eLRR or LRR kinase superfamilies and provide a lot of information about the structure and function of *R* genes that exhibit resistance response against a variety of pathogens such as virus, bacteria, fungi, nematodes and pests. These and other domains like TIR and WRKY have been reported to play a major role in signal transduction. The present chapter focuses on *R* genes, structure of *R* proteins and perception of signal at early stages of *R–Avr* interaction to signal transduction.

Keywords

Disease resistance • *R–Avr* • Resistance genes

B.A. Padder (✉)
Plant Virology and Molecular Plant Pathology Laboratory, Division of Plant Pathology, Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir, Shalimar, Jammu and Kashmir, India
e-mail: bapadder@rediffmail.com

20.1 Introduction

Plant diseases cause huge losses to growers every year worldwide (Agrois 1988) and have been present from the beginning in an organised agricultural system. In contrast to vertebrate

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immune system where specialised cell devoted to defence are mobilised to site of infection to kill or limit the growth of invading pathogen, the lack of circulatory system in plants requires a strategy where each cell has to defend itself from infection (Dangl and Jones 2001; Holub et al. 2001; Jones 2001). Being sessile, plants are constantly challenged by variety of pathogens, but the disease development is rear (resistance is the rule and susceptibility is exception). Over the course, plants have developed an array of mechanisms to avoid pathogen attack thus exhibiting resistance to majority of pathogens: either (1) plant does not support the nutritional requirements for pathogen (non-host resistance) (Heath 2000) or (2) plants possess preformed structural barriers or preformed toxic compounds of various nature warding off majority of pathogens but specialised pathogens can successfully invade and cause disease or (3) a sophisticated mechanism by triggering an effective innate immunity response after perceiving pathogen attack (resistance genes) (Beynon 1997). In this chapter, first gene-for-gene concept then models of *Avr–R* gene interaction and later how signal transduction cascade elicits resistance response are discussed.

20.2 Plant Disease Resistance Genes and Gene-for-Gene Interaction

Sixty years ago, Flor, while working with flax and flax rust interaction, found genotypes of flax showing differential resistance response when inoculated with different pathogen isolates. These genetic studies were carried out by crossing resistant and susceptible host genotypes and crosses of fungal isolates that differed in their ability to infect a particular genotype (Flor 1971) with the conclusion that resistance response in flax is inherited in a simple Mendelian fashion. Such genetic experiments by Flor and Oort working independently made a breakthrough which led to gene-for-gene concept, stating that for every dominant gene (*R* gene) determining resistance in the host, there is a corresponding dominant gene for avirulence (*Avr* gene) in the pathogen (Fig. 20.1). Since then comprehensive knowledge has been accumulated in various plant–pathogen interactions exhibiting classical gene-for-gene interaction (Whalen et al. 1991; Sharma et al. 2009; Nair and Thomas 2007; De Wit 2007; Keen 1992, 1997).

The crux of defence mechanism in plant pathogen interaction is recognition at molecular level. If the host cell fails to recognise an invader, it will also fail to mount a defence response and become diseased. On the other hand, recognition of potential pathogen by the plant would lead to the activation of defence reaction in plant and prevent disease. These interactions require that a plant contain a resistance (*R*) gene and a corresponding avirulence (*Avr*) gene be present in the invading pathogen. These *R* and *Avr* genes are generally inherited as dominant characters in plant and pathogen, respectively. If either partner lacks a functional allele recognition, resistance does not occur. A plant may have a number of different *R* genes directed to a particular pathogen, and similarly, a pathogen may have more than one *Avr* gene. Thus, to escape surveillance, the pathogen must possess recessive alleles for all the relevant *Avr* genes.

R genes are abundant in plant genomes and in most cases belong to tightly linked gene families with diverse recognitional specificities (Meyers et al. 1999; Ye and Ting 2008; Xiao et al. 2001; Whitham et al. 2000; Zhou et al. 1995, 1997; Holub et al. 2001). More than 50 *R* genes with different recognition specificities have been characterised from monocots and dicots and are effective against a broad spectrum of pathogens (Table 20.1). These genes have three main surveillance functions in the plant cell: (1) to detect *Avr* gene-encoded molecules, (2) to initiate signal transduction for activation of defence mechanisms and (3) to evolve rapidly in order to recognise new pathogens or variants of old ones that have lost the originally recognised *Avr* gene products (Hammond-Kosack and Jones 1996, 1997; White et al. 2000; Piedras et al. 1998). Most of resistance genes described so far are either involved in perception or signal transduction with the exception of *Hm1* gene as *R* genes encode proteins with some common motifs playing pivotal role in

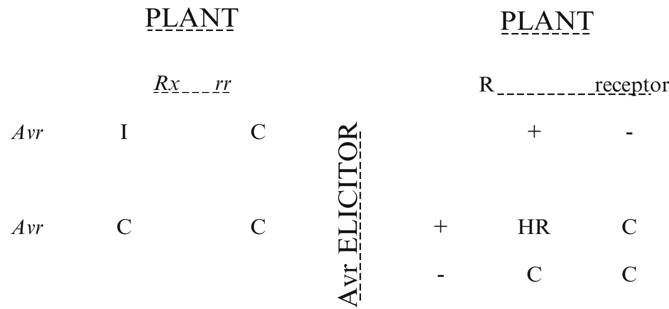

Fig. 20.1 Alternative alleles of a disease resistance gene (R or r) and the complimentary pathogen avirulence gene (*Avr* or *avr*); only the occurrence of both dominant alleles results in incompatible interaction (*left*). The elicitor of pathogen on recognition by plant R receptor activates the hypersensitive (*HR*) response and the *Avr* elicitor becomes a virulence factor in the absence of recognition (*right*)

Table 20.1 Classes of disease resistance genes in plants

Class	Domains	Host–pathogen interaction	Genes
Ia	TIR–NBS–LRR	*Arabidopsis–Peronospora parisitica*	*RPP1; Rpp10; Rpp14; RPP4; RPP4; RPS4*
		Arabidopsis–Pseudomonas syringae pv. *maculicola*	*SS14*
		Flax–*Melamsporalini*	*L6; M; P; P2*
		Tobacco–Xanthomonas campestris	*Bs4*
		Tobacco–Tobacco mosaic virus	*N*
Ib	CC–NBS–LRR	*Arabidopsis–Pseudomonas syringae*	*RPS2; RPM1*
		Tomato–*Pseudomonas syringae*	*RPM1*
		Tomato–*Fusarium oxysporum*	*I2*
		Tomato–*Meloidogyne javanica*	*Mi-1; Mi-9*
		Tomato–*Tomato spotted wilt virus*	*Mi*
		Rice–*Magnaporthe grisea*	*Pib; Pi-ta; Pi36*
		Rice–*Xanthomonas oryzae*	*Xa1*
	LZ–NBS–LRR	*Arabidopsis–Pseudomonas syringae*	*RPS5*
Ic	WRKY–NLS–TIR–NBS–LRR	*Arabidopsis–Ralstonia solanacearum*	*RPS1*
II	LRR–TMs	Tomato–*Cladosporium fulvum*	*Cf2, Cf4, Cf5; Cf9*
III	Protein kinase	Tomato–*Pseudomonas syringae* pv. *tomato*	*Pto*
IV	LRR–TM kinase	Rice–*Xanthomonas oryzae* pv. *oryzae*	*Xa21*
V	*HSPro1*	Beet–*Heterodera* sp.	*HSPro1*
VI	CC–LRR	*Arabidopsis–Erysiphe*	*Rpw8*
VII	TMs	Barley–*Blumeria* sp.	*mlo*
VIII	Toxin reductase	Maize–*Cochliobolus carbonum*	*Hm1*

perception and signal transduction (Ade et al. 2007; Baker et al. 1997; Zhou et al. 1998, 2000, 2001).

The predicted products of disease resistance genes can be classified into eight classes: NBS–LRRs, LRR–TMs, protein kinases, LRR–TM kinases, membrane proteins, toxin reductases and the other two have unique products (Hulbert et al. 2008).

The first and the largest group of resistance genes carry the leucine-rich repeats (LRRs) and nucleotide binding site (NBS) domains (Jones 2001; Kohler et al. 2008; Meyers et al. 1999).

The NBS–LRR class of *R* genes can be further subdivided based on their ability to code for other recognisable domains. One subclass codes for a TIR domain (*Drosophila Toll* and mammalian *interleukin*-1 receptors) at the *N* terminus of the protein and the other is without a TIR domain and codes for a coiled-coil (CC) structure near their *N* terminus, sometimes in the form of a leucine zipper (LZ) (Pan et al. 2000). This class contains disease resistance genes from diverse plant species such as flax (*L, M* and *P*), tobacco (*N*), Arabidopsis (*RPP1* and *RPP5*), pepper (*Bs2*), tomato (*I2. Mi*) and rice (*Pib, Pita*) (Hulbert et al. 2008). For the NBS–LRR class of *R* genes, the NBS and either the LZ or TIR domains are the most likely to be involved in signalling. The presence of NBS suggests that although these *R* proteins do not possess intrinsic kinase activity, they could activate kinase or G proteins. The leucine zipper regions found in *Rpm1*, *RPS2* and *Prf* which in each protein precede the NBS, potentially could facilitate homodimerisation of the proteins themselves or heterodimers with other proteins. *R* proteins could exist as monomers before pathogen challenge and then undergo dimerisation or oligomerisation upon activation. Alternatively, they may exist initially as dimers or multimers that dissociate upon activation (Hammond-Kosack and Jones 1997). Arabidopsis resistance gene *RRS1* conferring resistance to the bacterial phytopathogen *Ralstonia solanacearum* is a new member of the NBS–LRR class. In addition to NBS–LRR, it contains TIR, a putative nuclear localisation signal (NLS) and a WRKY domain (Deslandes et al. 2002). The WRKY domain has a conserved amino acid sequence WRKYGQK at its *N*-terminal end, along with a novel zinc-finger-like motif.

The second group, i.e. LRR–TMs, includes *Cf2*, *Cf4*, *Cf5* and *Cf9* genes conferring resistance to *Cladosporium fulvum* race containing the corresponding *Avr* genes. These *Cf* genes encode putative membrane anchored extra-cytoplasmic glycoprotein and show homology to the receptors domains of several receptor-like protein kinases and to members of the LRR family of proteins. The *N*-terminal contains a putative signal peptide followed by an LRR domain with 33 perfect and 5 imperfect repeats. The LRR domain is followed by a hydrophobic stretch of amino acids consistent with membrane-spanning domain region. The similarity in structure of the tomato *Cf* proteins (LRR–TM class) to the *Xa21* (LRR–TM kinase) suggests that the LRR–TM genes may also include a kinase in their defence-signalling pathway.

The third group includes the tomato *Pto* gene, conferring resistance to *Pseudomonas syringae* pv. *tomato* containing the *avr* gene. *Pto* belongs to a multigene family encoding proteins with similarity to serine–threonine protein kinase (STK), which suggests a role of *Pto* in cellular signalling via protein phosphorylation. The gene contains no signal sequence, LRR structures or membrane-spanning domain. This suggests a cytoplasmic location for the gene product, but the *N*-terminal region does contain a potential myristoylation site that may imply that the protein is in fact membrane associated (Beynon 1997). It has been observed previously that plants carrying *Pto* gene were sensitive to the application of the organophosphate insecticide fenthion and produced small necrotic spots similar to those produced in the HR. Analysis of the *Pto* locus showed that one of the other kinases (*Fen*), and not *Pto* itself, was responsible for the sensitivity to fenthion. Hence, the genes *Pto* and *Fen* produce a similar plant response on exposure to very different stimuli, a plant pathogen and a synthetic chemical, respectively. This shows that the detection mechanisms of plant are not limited to proteins and allow the plant to respond to secondary metabolites produced by the pathogen.

The fourth group, i.e. LRR–TM kinases, includes rice *Xa21* gene, conferring resistance to *Xanthomonas oryzae* pv. *oryzae*, encoding a receptor kinase-like protein. This class of proteins is predicted to span the cell membrane, with an extracellular LRR (Hulbert et al. 2008). Compared with previously cloned genes, the structure of *Xa21* represents a novel class of plant disease *R* genes encoding a putative receptor kinase (RK) (Ronald 1997). *N*-terminal domain is targeted to an extracellular location. This is followed by 23 imperfect copies of a 24 amino acid leucine-rich repeats (LRR). LRR motifs have

been implicated in protein/protein interactions. After the LRR, the protein contains a structure likely to be a membrane-spanning helix, suggesting that although *N*-terminal of the protein is extracellular, the *C*-terminal is intracellular.

The fifth class included *HSpro-1* R gene conferring resistance in Beet to *Heterodera* sp. The predicted *HSpro-1* protein originally reported to have an LRR–TM signature though it poorly fits the LRR consensus and has minimal similarity to other known resistance genes (Ellis and Jones 1998).

The sixth class includes *Rpw-8* gene which confers resistance in *Arabidopsis* against *Erysiphe*. The seventh group includes the barley *mlo* gene, conferring resistance to *Blumeria* sp., which produces 60 kDa protein in which the membrane is anchored with at least six membrane-spanning helices (Hulbert et al. 2008).

The *Hm1* conferring race-specific resistance to the fungal pathogen *Cochliobolus carbonum* race 1 represents the eighth class. The gene was isolated using a transposon-tagging method and encodes an NADPH-dependent toxin reductase which inactivates the HC toxin produced by the fungus (Johal and Briggs 1992).

20.3 Models for Gene-for-Gene Interaction

The ability to detect the presence of pathogen is a prerequisite for plant cell to respond promptly to invading pathogens. In order to elicit resistance response, the plant and pathogen need to express matching genes at interface, i.e. the plant *R* gene and pathogen *Avr* gene. Though *R–Avr* interaction is now firmly supported by characterisation of more than 40 *R* genes from diverse plants and *Avr* genes from various pathogens. However, the underlying perception mechanism has been a subject of debate and many models were put forth to explain the interaction. Most of these models at the host–pathogen interface are based on the *Avr* protein (*Avr* effector/elicitor) which is recognised by *R* protein either directly or indirectly.

A simple model for gene-for-gene is based on direct physical interaction (Fig. 20.2a) between *Avr* effector and *R* protein and is classically known as ligand–receptor model (Keen 1990). The *Avr* effector (ligand) directly binds to *R* protein (receptor); the *Avr–R* complex initiates a cascade of reaction leading to resistance in the plants and where *Avr* effector is not recognised by *R* gene leads to susceptibility. A few complementary gene interactions exhibiting direct physical interaction are *Pita–AvrPita*, *Pto–AvrPto* and many more; the physical interaction of their products induces hypersensitive reaction inside plant cells (Scofield et al. 1996; Jia et al. 2000). However, in many host–pathogen interactions, the *R–Avr*-encoded proteins have not shown direct physical interaction which led to the 'guard model' proposed by van der Biezen and Jones (1998) to explain the indirect interaction between *Pto–AvrPto* and *Prf*. Basically the extension of ligand–receptor model mainly explaining how single *R* gene-encoded protein recognises multiple *Avr* effectors from diverse pathogens in the presence of other host proteins 'gaurdee' responsible for initiation of resistance response (Fig. 20.2b). According to the guard model, *Avr* effector mainly functions as virulence factors, and the pathogenicity targets affected by the effector could be the proteins involved in defence or in host metabolism. *R* protein binds to the *Avr*–guard protein complex and elicits defence response in the host; for instance, *Pto* linked *Prf* recognises *AvrPto–Pto* complex and activates pathways leading to defence response. Similarly *Avr*1 protein from bacterium *Pseudomonas savastanoi* pv. *maculicola* does not directly bind to corresponding *R* protein and instead binds to RIN4, and the other classical examples are *PBS1* in Arabidopsis and *RCR3* and *Pto* in tomato (Jones and Dangl 2006). The main drawback of this model are as follows: Firstly, that gaurdee protein does not play significant role in the absence of *R* gene-encoded protein, and the interaction of gaurdee with *Avr* effector does not enhance pathogen fitness. Secondly, the selection pressure in the absence of *R* protein decreases the binding affinity of gaurdee and thus evades detection and modification of effector, while in presence favours gaurdee to improve the interaction and enhance perception. In order to explain

such a phenomena, recently van der Hoorna and Kamoun (2008) proposed the ‘decoy model’. According to this model (Fig. 20.2c), the effector target monitored by the *R* protein is a decoy that mimics the operative effector target but only functions in perception of pathogen effectors without contributing pathogen fitness in the absence of its cognate *R* protein. Decoy is a host-encoded protein which helps in the perception of *Avr* effector, but itself has no role in the development of disease or elicitation of resistance response in the host.

Since most of the *R* genes belong to NBS–LRR class within a subgroup STAND (*s*ignal *t*ransduction *A*TPases with *n*umerous *d*omains) family, Lukasik and Takken in 2009 proposed the ‘mechanistic model’ (Fig. 20.2d). The model is basically the extension of the above-mentioned models which describes the interaction dynamics and specific functions of the individual subdomains of mainly NBS–LRR *R* gene class in signalling. In the mechanistic model, CC and LRR domains bind the NB–ARC, thereby providing an interaction platform that mediates

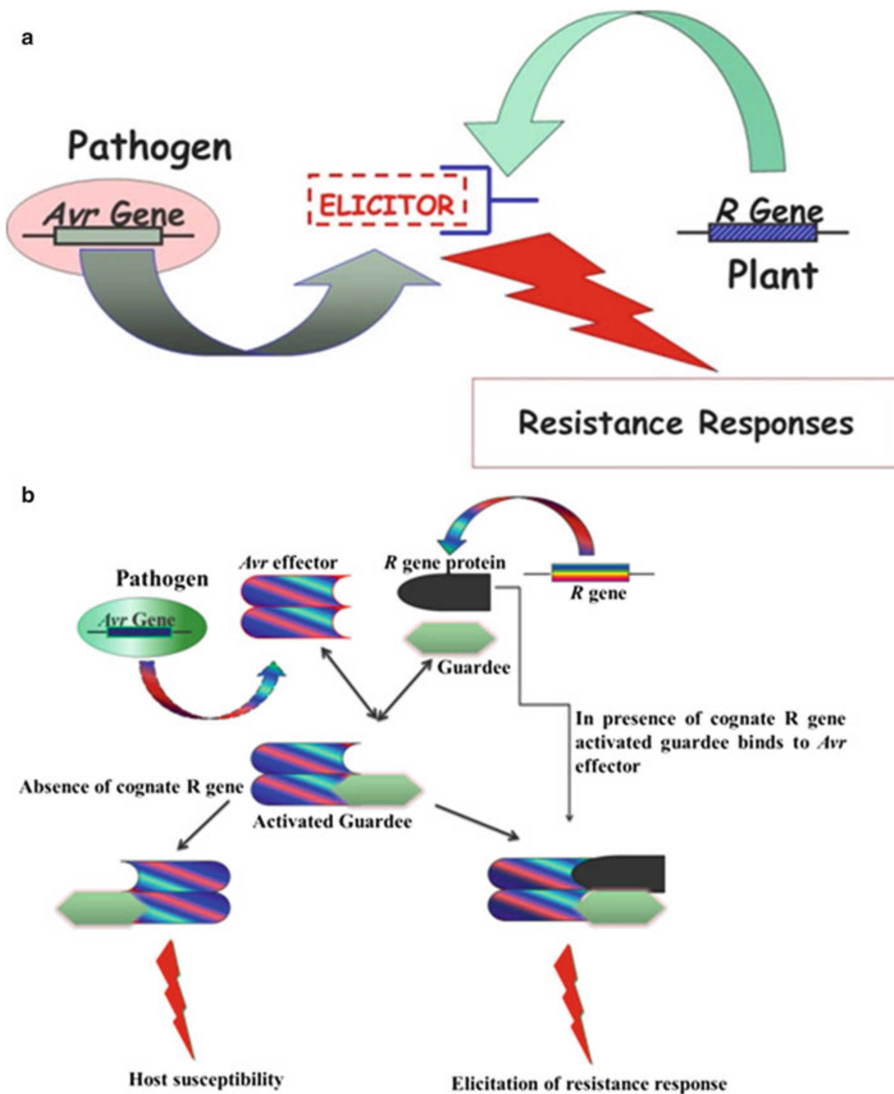

Fig. 20.2 Different models of gene-for-gene concept: (**a**) ligand receptor model; (**b**) guard model; (**c**) decoy model; (**d**) mechanistic model

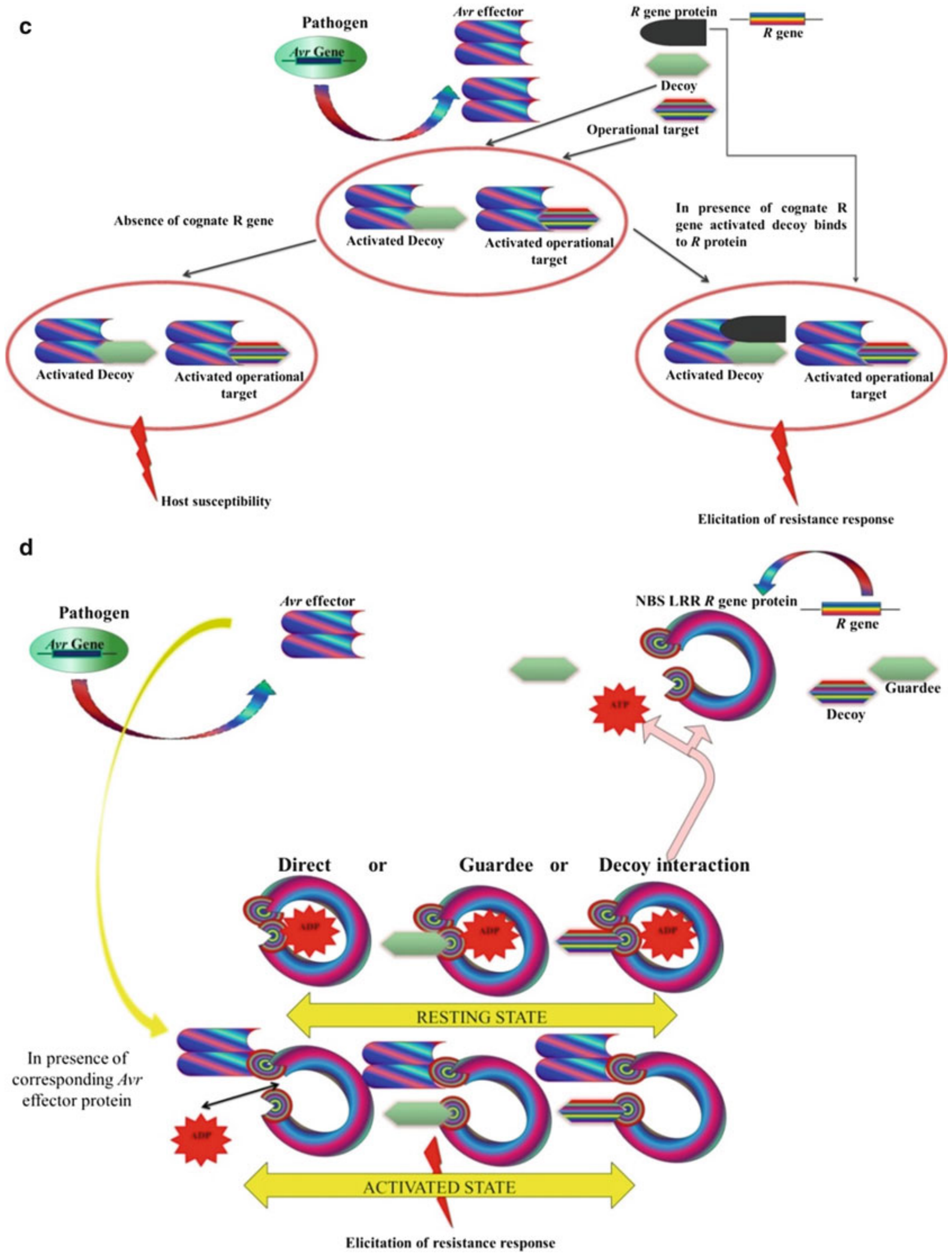

Fig. 20.2 (continued)

Avr recognition at their interface. Such a closed conformation stabilises the *N*-terminal part of the LRR domain, and NB–ARC domain of NB–LRRs functions as a molecular switch wherein the ADP-bound state represents the 'off' and the ATP the 'on' state.

20.4 R-Gene-Mediated Signalling

Multicellular organisms maintain homeostasis in their cells by recognising an extracellular substance which can be any biochemical substance or physical exposure, and the process of recognition is carried out using the so-called signalling systems. Effectors of plant pathogenic microorganisms have been found to induce a cascade of defence responses in the plant cell before the exhibition of resistance or susceptibility. Thus, signalling systems actually determine the early host–pathogen responses resulting either in compatible or incompatible interaction. The signalling systems at plant cell interface begin functioning when plant pathogen effector is recognised by host either directly or indirectly.

Considerable research during the last decade using mutational analysis in various plants has shown various loci necessary for R gene signalling (Parker et al. 1996; Peart et al. 2002; Warren et al. 1999). These genes are called RDR (*r*equired for *d*isease *r*esistance) and have been identified in many plants, for instance, in tomato 5 PDR genes *Rcr1* to *Rcr5* and barley Ror1 and Ror2 are necessary for elicitation of defence response by genes *Cfs* and *mlo*, respectively. One more important result from these mutational studies was the identification of *eds1* (*e*nhanced *d*isease *s*usceptibility) mutant in *Arabidopsis* which resulted in loss of race-specific resistance specified by *RPP5* resistance gene. The EDS1 protein shows similarity to lipases of eukaryotic organisms and is a key component in the initiation of defence response in plants containing TIR–NBS–LRR against many pathogens. The other protein required for proper functioning of TIR–NBS–LRR-type resistance genes is PAD4 (*p*hytoalexin deficient) and is involved in salicylic acid signalling pathway. Feys and Parker (2000) reported dimerisation and heterodimerisation of EDS1 with PAD4; hence, both the proteins are necessary for accumulation of signalling molecule salicylic acid thus performing distinct functioning in defence-signalling pathway. EDS1 is an essential component for elicitation of hypersensitive reaction especially the early reactive oxygen burst, while PAD4 is required to strengthen the hypersensitivity or does activity downstream of hypersensitivity initiation. Furthermore, the level of salicylic acid accumulation and its activity is regulated by FMO1 monooxygenase and NUD7 hydrolase (Bartsch et al. 2006). The necrotrophic fungus *Botrytis cinerea* manipulates plant signal transduction pathway by the activation of the *EDS1* and *SGT1* genes. Similarly, in *A. thaliana*, *SGT1* regulates the resistance processes initiated by *RPP2*- and *RPP4*-encoded proteins. Interaction of SGT1 protein with HSP90 (heat-shock protein 90) has been reported essential for the resistance response, and both the proteins are involved in building a recognition complex consisting of R and RAR1 proteins and recognised effectors (Schulze-Lefert 2004; Boter et al. 2007).

Functioning of resistance genes encoding CC–NBS–LRR/LZ–NBS–LRR proteins requires a product of another gene NDR1 (*n*o *d*isease *r*esistance) for elicitation of defence response. The NDR1 gene encodes a small, highly basic and putatively integral membrane protein required at an early stage of signal transduction mainly at the generation of reactive oxygen species and salicylic acid accumulation. In many NBS–LRR genes with CC or LZ domains like *PBS2 and RPT2*, NDR1 is essential for disease resistance; however, certain genes like *RPM1* can elicit hypersensitive response even in the absence of NDR1, but the growth of pathogen carrying corresponding *Avr* gene is not restricted (Century et al. 1997). Recently Day et al. (2005, 2006) reported that the interaction of *RPM1* and *RPS2* encoded proteins with RIN4 but, for initiation of resistance response, requires two different pathways. The interesting protein Npr1 (non-expressor of PR genes) plays a dual role, i.e. salicylic acid and jasmonic acid-/ethylene-mediated signalling

pathways. The protein Npr1 resembles to a typical ankirines protein that helps in protein–protein interaction particularly the TAG transcript factor family.

Earlier it was thought that defence signalling in plants exists in the cytoplasm; however, with the identification of nuclear localisation signal (NLS), WRKY- and WRKY-like activity domains in many R gene-encoded proteins indicate *R* proteins can also function in the nucleus (Burch-Smith et al. 2007; Ulker and Somssich 2004; Eulgem and Somssich 2007). Some of the WRKY transcriptional factors have been found to work as negative regulators of resistance-related gene expression. For instance, overexpression of chilli CaWRKY1 results in enhanced susceptibility towards pathogens in transgenic tobacco; similarly silencing of Arabidopsis WRKY11 and WRKY17 results in enhanced pathogen resistance (Journot-Catalino et al. 2006; Oh et al. 2008).

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About the Editors

Dr. Khalid Rehman Hakeem
Dr. Hakeem is currently working as Fellow Researcher at Faculty of Forestry, Universiti Putra Malaysia, Serdang, Selangor, Malaysia. He was awarded PhD (Botany) in 2011 by Jamia Hamdard, New Delhi. Dr. Khalid has more than 7 years of teaching/research experience in plant physiology biotechnology, as well as ecology. Recipient of several fellowships at both national and international level, Dr. Khalid has so far edited more than seven books with Springer and other European publishers. He has also to his credit more than 30 publications in various national and international journals.

Dr. Reiaz Ul Rehman
Dr. Rehman is currently working as an assistant professor, Department of Bioresources, University of Kashmir, Srinagar, India. He was awarded PhD (plant biotechnology) in 2003 by Jamia Hamdard, New Delhi. Dr. Reiaz ul Rehman has about 14 years of teaching/research experience in plant biology. He is a recipient of several fellowships at both national and international level, particularly, the award of the Marie Curie IIF project (MC-IIF-FP7-219339). He has worked on agronomical, physiological and biochemical aspects of mineral nutrition in mustard, which is relevant to economical aspect in terms of crop productivity. He managed the in vitro regeneration and propagation of several important medicinal plants followed by the genetic transformation of medicinal plants with stress-related genes. During his postdoctoral research, he worked in the field of endomembrane biology and established the role of certain proteins during polarized growth of pollen tubes and the mechanisms regulating ion influx at the plasma membrane and their role in membrane secretion. He has to his credit one published book and 22 publications in various national and international journals.

Dr. Inayatullah Tahir
Dr. Tahir is currently working as a professor, Department of Botany, University of Kashmir, Srinagar, India. He earned his PhD degree in botany from Kashmir University in 1986. Prof. Inayatullah Tahir has more than 28 years of teaching and research experience. He has done extensive work pertaining to physiological studies on buckwheats in Kashmir. Buckwheat belonging to genus Fagopyrum is an important bioresource in view of its historical perspective and socio-economic benefits. He has also been actively perusing the research on flower senescence and postharvest physiology of the ornamentals. He has published more than 60 papers in various national and international journals. He has attended and presented his research findings in various national and international conferences; besides, he is life member of several learned scientific societies.

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